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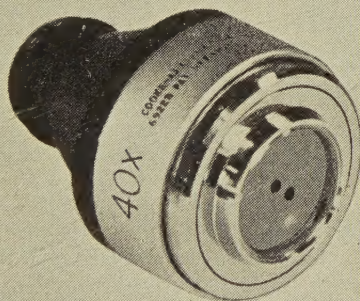
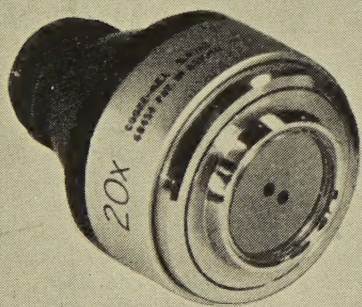
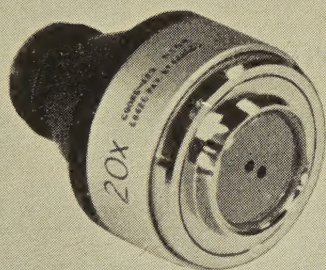
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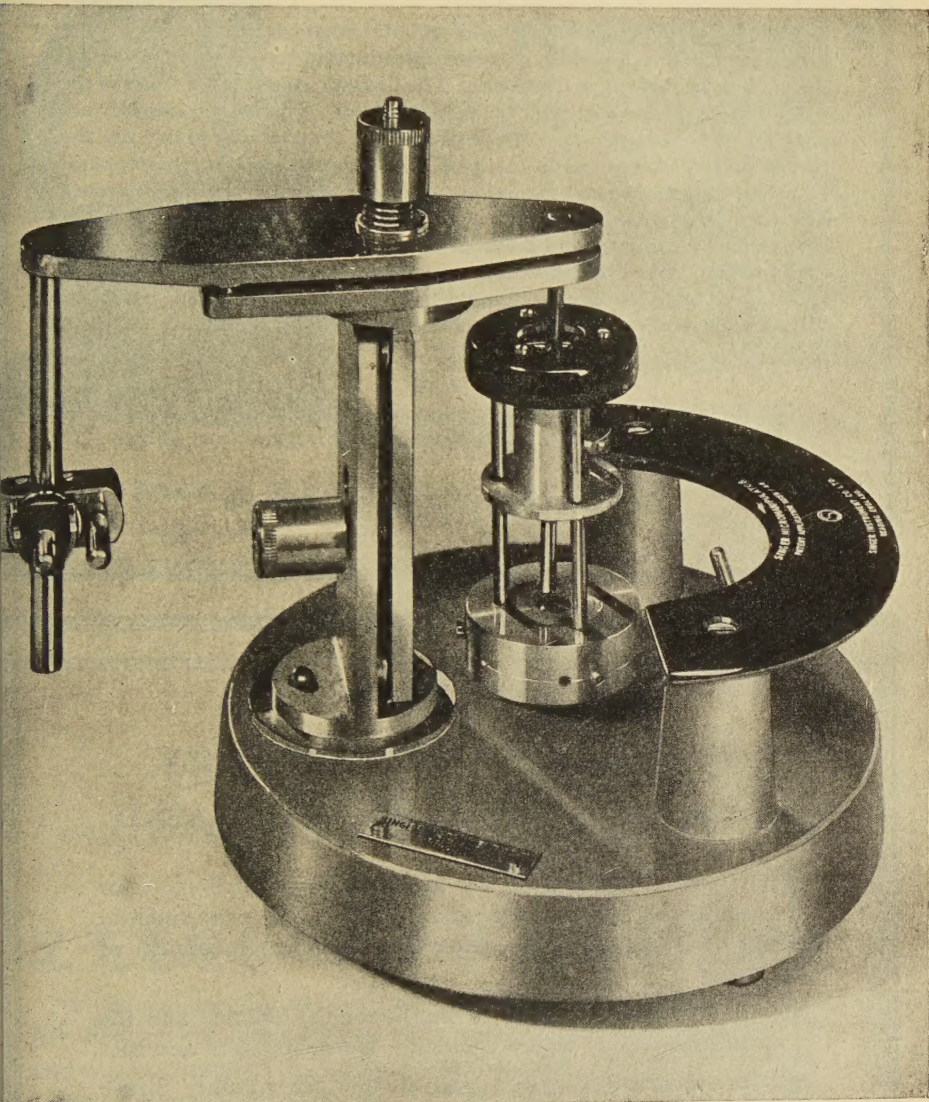
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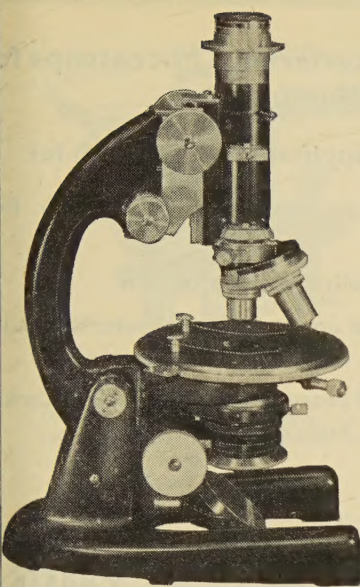
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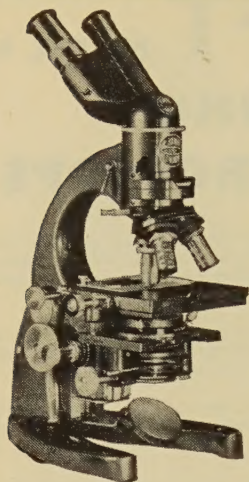
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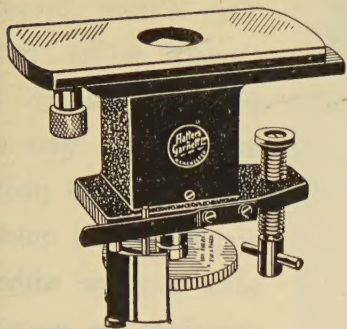
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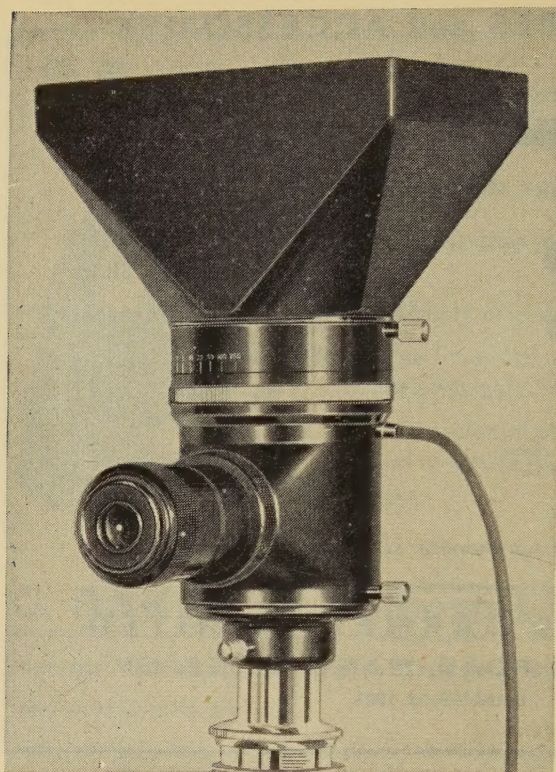
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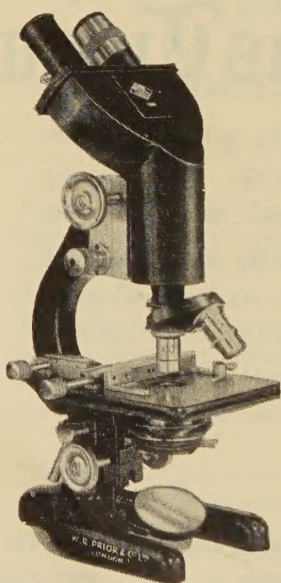


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The Structure and Hydromechanics of the Musculo-epithelium in *Metridium*

By ELAINE A. ROBSON

(From the Zoological Laboratory, Cambridge)

With 2 plates (figs. 1 and 3)

SUMMARY

Musculo-epithelial cells have been isolated from mesenteries of the sea-anemone *Metridium senile*, and the descriptions of earlier workers confirmed. The cells contribute both to the muscle-field above the mesogloea and to its overlying epithelium. In sections or whole mounts it is possible to see numerous vertical strands passing from the epithelial elements to their muscle-fibres. The protoplasmic strands are separate from one another and are thus surrounded by fluid which forms a continuous thin layer between the epithelium and muscle-field. It is proposed to call this the subepithelial fluid.

Epithelial elements from contracted mesenteries are much taller than those from stretched tissue. As the area of the mesentery decreases during contraction a reversible change from pavement to columnar epithelium takes place. The epithelium is able to follow rapid contractions without delay, owing to the hydrostatic action of the subepithelial fluid in thrusting it outward. There is as yet no evidence that the epithelial protoplasm moves by its own activity during contraction or relaxation. It may be moved passively and has considerable elasticity. Modifications of the musculo-epithelium in certain anatomical regions are discussed.

Although true musculo-epithelium characterizes only the coelenterates, analogous systems occur in the tissues of several higher animals, and it is suggested that intercellular fluid may have a hydrostatic function in these situations also. The possible metabolic role of subepithelial fluid in *Metridium* is discussed, and it is suggested that the subepithelial fluid and the mesogloal fluid together form an 'internal medium' which may provide some degree of biochemical co-ordination in this animal.

INTRODUCTION

METRIDIUM SENILE is an acontiarian sea-anemone (Stephenson, 1935), whose behaviour and anatomy have received considerable study (Parker, 1919; Parker and Titus, 1916; Batham and Pantin, 1950 *a-c*, 1951, 1954; Pantin, 1952). Its normal activity has a much slower time-scale than that of higher animals, and an inactive appearance may conceal continuous changes in shape which, although extensive, are too slow for the eye to follow. The movements observed in prolonged cycles of activity and in immediate reactions are due to the interplay of various sets of muscles, which contract against the slight pressure of the coelenteric fluid and cause the animal to lengthen or to shorten accordingly. While the nervous and sensory systems play a large part in this activity and must be considered in any physiological interpretation of sea-anemone behaviour, the contractile properties and structure of the muscles are equally important in the living animal. Batham and Pantin's work in this field has already thrown light on many aspects of the muscular

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system in *Metridium*. The cells of this primitive tissue are organized on a basis different from that in higher animals, and as they have not yet been examined from a functional point of view, it is now proposed to do so.

Since *Metridium* is diploblastic, the muscles develop in single layers over various anatomical surfaces. The fibres run parallel, forming the extremely drawn-out lattice work known as a muscle-field (Batham and Pantin, 1951). This can give rise to more elaborate muscles only by folding, as in the mesenteric retractors, or by rolling up into cylindrical bundles which penetrate the mesogloea, as in the sphincter (fig. 3, c). The muscle-fibres can contract to a quarter or less of their extended length, and appear to be restricted in their excursions by the special properties of the mesogloea immediately beneath (Batham and Pantin). The rest of the mesogloea is strikingly extensible owing to its fibrous crossed-lattice structure (Chapman, 1953). Above the muscle-field lies an epithelium which has been little studied, although it is able to follow large and occasionally rapid contractions and must possess interesting properties as a plastic covering layer. The organization of the epithelium and its relation to the underlying muscle-field will be considered below.

THE STRUCTURE OF MUSCULO-EPITHELIUM

As long ago as 1879, the brothers Hertwig showed that muscle-fibres in the endoderm of actinians were really part of epithelial cells, and one of their illustrations is reproduced in fig. 1, A. The cell-body of each muscle fibre was found in the epithelium, and the two layers formed one system. The Hertwigs, interested in the evolutionary aspects of cell-structure, pointed out that this primitive condition could be modified by stages in which the muscle-fibre was developed at the expense of the rest of the cell. In the large muscle-fibres of the sphincter, sunk into the mesogloea (fig. 3, c), the cell-body is reduced to a nucleus and a small mass of cytoplasm. The same is true of the ectodermal muscles of the disk and tentacles in *Metridium*, and in this case separate

FIG. 1 (plate). A, musculo-epithelium from a mesentery of *Sagartia* (= *Calliactis*) *parasitica* as drawn by the brothers Hertwig (1879, Taf. VI, fig. 8).

B, small musculo-epithelial cell, isolated from a stretched *Metridium* mesentery. Hertwig maceration, picrocarmine.

C, similar cell from more contracted tissues. Hertwig maceration, picrocarmine.

D, field of flagella from stretched mesentery. Baker's formaldehyde-calcium, iron haematoxylin.

E, epithelial flagella and their basal granules under high magnification. Baker's formaldehyde-calcium, iron haematoxylin. White lines are cracks in the haematein lake.

F, basal granules and epithelial cell boundaries in another stretched mesentery. The dark mass is a mucus cell. Baker's formaldehyde-calcium, iron haematoxylin.

G, connexions between muscle-field (below) and epithelium in whole mount of a mesentery. The epithelium (upper right) has been pulled apart. Baker's formaldehyde-calcium, iron haematoxylin.

H, connexions between living epithelium (top) and muscle-field (below) in fresh *Metridium* mesentery. Compare with (g) and (i). Preparation considerably stretched.

I, section of a stretched mesentery showing the musculo-epithelium: note protoplasmic strands. Muscle-fibres are cut through transversely, and lie just above the wavy mesogloea fibres. Flemming-without-acetic, haemalum eosin.

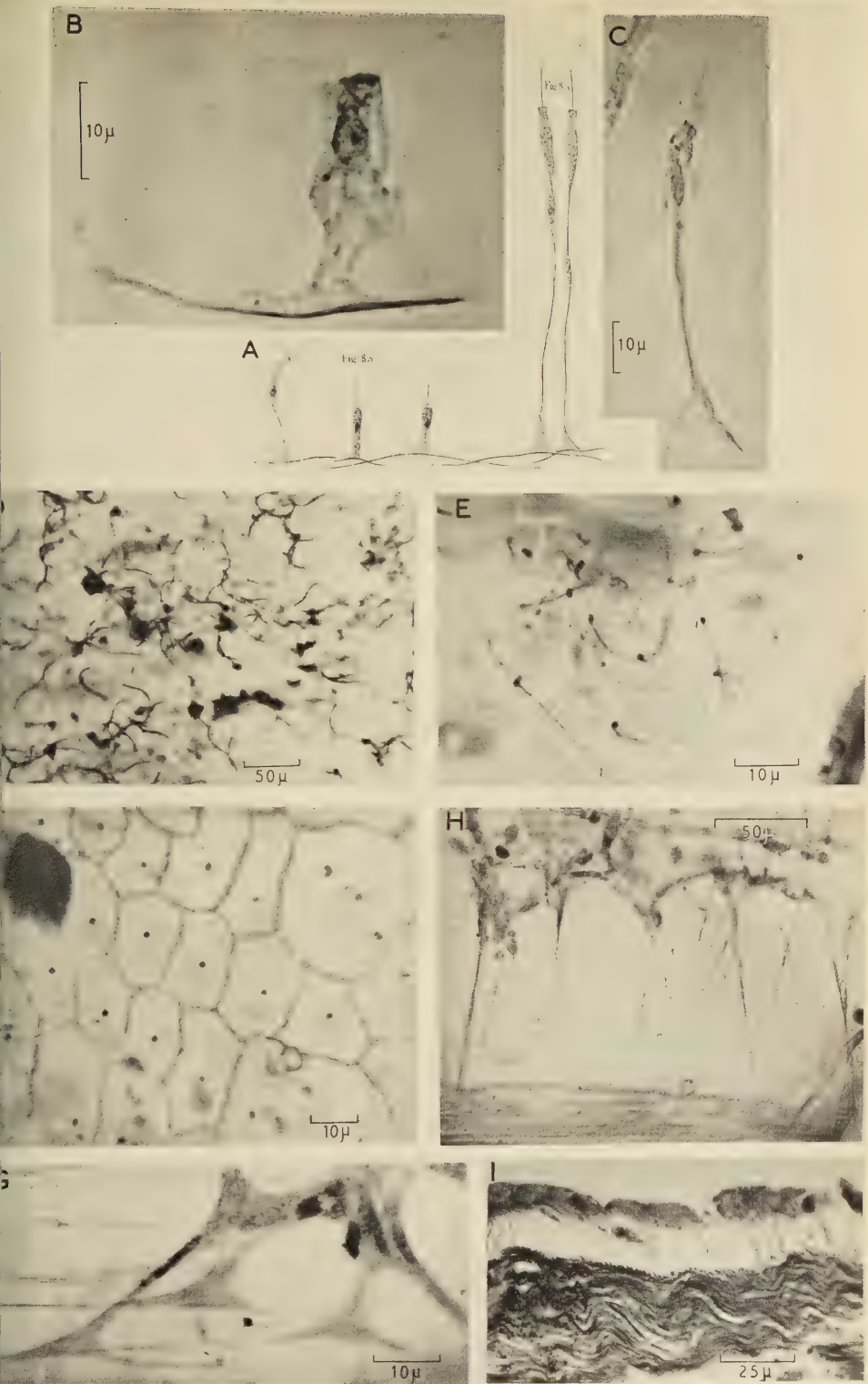


FIG. 1
E. A. ROBSON

epithelial cells provide a covering layer. True musculo-epithelium is present throughout most of the endoderm, however, and the question of immediate interest is its functional activity in these primitive metazoa. It is relevant to consider the fine structure of the cells, their attachment to each other and to the mesogloea, and their behaviour in living tissues during contraction. Later on it may be possible to approach the problem of morphogenesis and to find out how such a system develops, grows, regenerates when damaged, or represses during starvation. *Hydra*, for instance, possesses a reservoir of undifferentiated interstitial cells, but there is at present no suggestion of anything comparable in sea-anemones, and little is known of how new tissues arise.

The following account applies to cells from the mesenteries of *Metridium*, which provide endodermal tissues easy to prepare and to examine (see Batham and Pantin, 1951). The cells described by the Hertwigs can be isolated by their osmic-acetic technique, or by macerating with Goodrich's fluid (1942); small one from a stretched mesentery is shown in fig. 1, B. The epithelial part of the cell contains an oval nucleus in granular cytoplasm, and carries one long flagellum at its free surface. The base fans out as a crest along a refringent muscle-fibre, which is thus wholly invested with a fine layer of cytoplasm. In addition, small cytoplasmic processes may project irregularly beneath the muscle-fibre, which is probably attached by this means to the underlying mesogloea, and perhaps to its neighbours as well (this feature is not shown in fig. 1, B): Semal van Gansen (1952) makes a similar observation for *Hydra*. At least some of the small granules in the epithelial cytoplasm are mitochondria, for they stain with janus green B in living tissues (Lazarow and Cooperstein, 1953): similar bodies are again present in the endodermal cells of *Hydra* (Semal van Gansen 1954).

Contracted mesenteries yield musculo-epithelial cells which are very much smaller than the one in fig. 1, B, and a typically elongated cell of comparable size is shown in fig. 1, C. In very contracted tissues the epithelial part of the cell is even more attenuated, and the nucleus appears as a bead in a thread of cytoplasm. The portion bearing the flagellum usually remains slightly club-shaped. These changes were described by the Hertwigs and are those which might be expected if the epithelium accommodated to contraction of its underlying muscle-fibres by changes in height. (It should be noted, however, that fig. 1, A, reproduced from the Hertwigs, does not illustrate one cell in several phases of contraction, but shows the difference between cells from the crests and troughs of permanent folds in the retractor muscle. A figure of contracted and relaxed musculo-epithelium cells from a tentacle of *Sagartia* is given in Taf. VI, fig. 11, of the monograph.)

The features characteristic of musculo-epithelial cells can also be seen in whole mounts of *Metridium* mesenteries, especially after staining with Heidenhain's iron haematoxylin (Pantin, 1946). The iron haematein lake is deposited in almost every contour, and suitable differentiation of a whole mount thus reveals both intracellular and surface structures. Baker's formaldehyde-calcium (1944) is a good fixative for these tissues.

Among the most evident characteristics of an intact mesentery is the forest of long flagella which stand out from the surface as shown in fig. 1, D. They may be $30\ \mu$ long and appear tangled or straight, depending probably on the speed of fixation. The basal granule of each may be seen just below the epithelial surface, as in fig. 1, E. In life every flagellum beats rapidly, and a current flows over the surface of the mesentery. Most of the flagella appear to lash backwards and forwards in one plane like cilia, but it is interesting to note that those which move slowly (whether naturally or as a result of damage is not clear) sometimes undulate instead. Neighbouring flagella are not co-ordinated, but all beat in the same direction.

It is customary to refer to the 'ciliary currents' of actinians, and the term 'ciliary reversal' is well known (Parker, 1919). These physiological attributes are, however, largely due to cells which possess only one long 'cilium' each. The musculo-epithelial cells are thus, strictly speaking, flagellated, and in this account the conventional word flagellum will be used. It seems difficult to distinguish between cilia and flagella on structural grounds (Bradfield, 1955), and there is probably little difference in their fundamental properties.

The cell boundaries of stretched mesenteries are also revealed by iron haematoxylin. The epithelium consists of polygonal units, each with its flagellum and nucleus, as in fig. 1, F. As has been seen in macerations, the dimensions of the epithelial elements vary with the state of contraction of the mesentery.

The muscle-fibres beneath the epithelium also stain well with haematoxylin, as shown by Batham and Pantin (1951). Individual fibres can be traced in the network of the muscle-field and usually meet with one another at their ends. It is unlikely that they are in cytoplasmic continuity, because when living tissues are treated with methylene blue, the fibres stain singly and not as a syncytium. The muscle-field nevertheless functions as a whole in many of the animal's activities, and the problem of how separate fibres combine to form a supracellular complex is an important one. There is no evidence so far that a true syncytium is present, although contraction often involves the whole tissue. Macerations show clearly the connexion between the epithelium and each fibre, however, as a more or less attenuated thread of protoplasm. This is also evident in whole mesenteries wherever a rent in the epithelium has exposed the muscle-field. The gap is then spanned by numerous protoplasmic stems connecting muscle fibres to their cell-bodies. One such region is shown in fig. 1, G, which may be compared with fig. 1, B. These features can also be distinguished in living tissues, and fig. 1, H shows muscle-fibres and their epithelium at the edge of a similar tear in a living, unstained mesentery. It illustrates the striking tensile properties of the musculo-epithelial 'stems' when stretched.

Sections of fixed material also show the link between muscle-fibres and the epithelium, as in fig. 1, I. It is well demonstrated in mesenteries cut slightly obliquely, especially when the epithelium happens to be slightly displaced during fixation.

A diagram attempting to relate the observations made on extended and contracted tissues is given in fig. 2. It is clear that the epithelial elements form a coherent mosaic and, by definition, each element is completely surrounded by its neighbours. The underlying muscle-fibres, however, form a latticework, and here each element runs separately for most of its length.

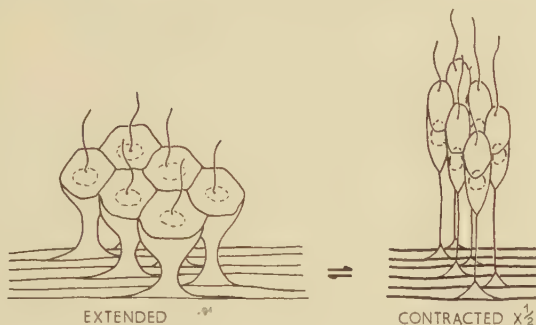


FIG. 2. Diagram representing movements of the musculo-epithelium (different parts not drawn to scale).

Protoplasmic connexions from the epithelial cell-bodies must therefore diverge as they approach the long, thin muscle-fibres. That is, owing to the geometry of the situation, the protoplasmic stems must lie in a space, and fig. 1, G-I, shows that in fact they do so. The musculo-epithelial cells are thus anchored above and below, while their central portions are free and lie in a space. Observation suggests that however small the space in this system may be, it extends continuously between the epithelium and the muscle-field. In living tissues such a space undoubtedly contains fluid. The physical and chemical properties of this subepithelial fluid would confer upon it several functions, of which one would be to act as a hydrostatic layer whenever the muscle contracts. These features do not appear to have been described from higher animals. They are discussed more fully below.

CHANGES ASSOCIATED WITH CONTRACTION

It seems likely that the four- or fivefold changes in length undergone by contracting muscle-fibres in *Metridium* will involve corresponding reductions in area of the overlying epithelium. It has been seen that the epithelial elements adapt themselves during contraction by becoming tall and thin, so that a reversible change from cubic to columnar epithelium takes place, as suggested in fig. 2. In this system the layer of subepithelial fluid also changes in depth because it is incompressible. As the extended muscle-field contracts, its decreasing area must cause a rise in pressure in the fluid layer: and although this will be transmitted in every direction, its only effect will be to thrust the cell-bodies outward, forcing them to elongate. The epithelium thus follows closely any contraction of the muscle-field, owing to the hydrostatic action of the fluid layer. In principle this mechanism will function whether the

volume of fluid present is large or small, but in practice the actual volume will probably affect the speed with which the epithelium accommodates because such fluid is less viscous than protoplasm. In rapid contractions of the whole tissue it would be an advantage for the volume of subepithelial fluid to be appreciable in relation to that of the epithelial protoplasm (see p. 273). The mechanical properties of the fluid layer would then enable the epithelium to function as a whole without excessive strains. This it appears to do, for instance, above the retractor muscle-fibres.

When fibres of the muscle-field itself are displaced by contraction of a reciprocal muscle, they accommodate by becoming buckled at right angles to the fibre axis (Batham and Pantin, 1951). The circular muscle of the tentacles or body-wall, for instance, becomes buckled whenever the longitudinal muscles contract. If contraction is maximal, or if adjacent muscle-fibres are not in the same state of tone, the epithelial layer also may become buckled. Conducted contractions, for example, may thus be accompanied by fine wrinkling of the epithelial surface, a condition which is often seen in the tentacles, where there is not much subepithelial fluid, and in other tissues such as the mesenteries. While it seems that the epithelium and muscle-field can buckle independently to a certain extent, the dynamics of the process have yet to be studied.

The hydrostatic function of the subepithelial fluid can only be maintained if it is sealed off from the external medium, and in this context the intercellular cement of the epithelium is an important contributing factor. In cases which have been investigated, intercellular cement appears to be part polysaccharide and part protein in nature (e.g., Essner, Sato, and Belkin, 1954), and this is probably true also of actinian material. The cell boundaries are revealed by Harmer's silver method (1884). They lend themselves to considerable stretching, and it seems likely that rapid epithelial changes are assisted also by elasticity of the cell surface. Micromanipulation with a glass needle certainly shows that fresh epithelium is very extensible and elastic. Also the decrease in total surface area of the mesentery which is associated with contraction could be achieved most rapidly if each epithelial cell bulged outwards as indicated in fig. 2: this suggestion accords with present observations.

When the muscle-fibres relax the epithelium returns smoothly to its original height. It is not clear whether the cell-bodies assist this process by shortening actively, and they are unfortunately too small to yield any useful information with polarized light. Relaxation is generally helped by the slight coelenteric pressure which acts on the whole animal. It is nearly always slower than contraction, and it is probably affected by viscosity of the protoplasm (and of the mesogloea) rather than by pressure changes in the subepithelial fluid.

It may be concluded from these observations that each musculo-epithelial cell forms part of a major effector system which functions as a whole in the living animal. A relatively small volume of fluid, forming a layer between the epithelium and muscle-field, possesses hydrostatic properties which become important during contraction. It enables the epithelial elements to follow

contracting muscle-fibres without delay and ensures that they are not distorted by excessive local strains. It may be noted that sensory, secretory, and other cells found together with musculo-epithelium will undergo parallel changes in shape. There is as yet no evidence that the epithelium plays an active part in re-extension, although the possibility should be borne in mind.

MICRO-INJECTION EXPERIMENTS

The presence of a subepithelial space in normal tissues has been confirmed by micro-injection experiments on living mesenteries. Coloured organic or mineral oil, introduced just beneath the epithelium with a sufficiently fine micro-pipette, does not pass through the muscle-field into the mesogloea, although it can spread quite a long way into the subepithelial space and distend the epithelium considerably. It is interesting that oil can also be injected into the mesogloea without difficulty, and its situation recognized at once. These observations have been made on mesenteries stretched over a glass window in a wax plate. A low-power objective can be used for examination.

Frozen sections of injected mesenteries (fixed in Baker's formaldehyde-calcium) confirm the presence of oil globules between epithelium and muscle, or in the mesogloea as the case may be. This method suggests that the subepithelial fluid occupies a fairly restricted space, even above the retractor muscle. The necessary measurements are difficult to obtain because the tissues vary in thickness, but in frozen sections of fresh or formalin-fixed contracted mesenteries, the subepithelium is itself at least $50\ \mu$ deep. These rough figures suggest that the fluid occupies perhaps 4% of the musculo-epithelium by volume. While it is true that sections of specimens prepared by various methods may show a subepithelial space so large that it represents a quarter or even half of the musculo-epithelium (fig. 1, I), this condition appears to be irregular rather than normal, and is probably caused by distortion of the tissues during treatment (compare fig. 1, H). Musculo-epithelium is developed in varying degrees in different parts of the anemone, but it is probably correct to estimate the volume of subepithelial fluid as less than 10% and more than 1% of the musculo-epithelium in most actinian tissues. The highest values are probably associated with rapidly conducting muscles such as the retractors and sphincter (see p. 273).

DISCUSSION

Before the organization of musculo-epithelium is discussed, let us consider some probable metabolic functions of the subepithelial fluid. Attention is drawn to the wandering cells which are present, often abundantly, throughout the epithelium and mesogloea, and almost invariably populate the subepithelial space. They are fairly small cells with round nuclei, and the cytoplasm is characteristically filled with fine droplets. The form of these amoebocytes is very variable in different preparations, but surprisingly uniform within one preparation, suggesting that the cells are of one kind only. This is seen in

fig. 3, A, showing part of a mesentery stained with chlorazol black E. Amoebocytes may sometimes aggregate locally, as illustrated by the section shown in fig. 3, B.

It was shown by Chapman (1953) that amoebocytes are probably not responsible for laying down mesogloal fibres, although their granulated cytoplasm suggests a secretory function. But they could very well have a role similar to those in Scyphomedusae, which Metchnikoff (1892) found to be concerned with wound-healing and tissue reorganization. They may form a physiological system which extends throughout the body of the sea-anemone. In this case, the intercellular fluid of the tissues, comprising not only the subepithelial layer but also the slightly hypertonic fluid of the mesogloea (see Chapman), could provide a continuous transport medium in which the amoebocytes might function. The passage of materials such as dissolved food and excretory products between endoderm and ectoderm, for example, and the reversible changes in the mesogloea which accompany growth, or regression during starvation, could perhaps be mediated by enzymes from these cells.

It is suggested that subepithelial fluid, while having a special hydrostatic function, is part of the general internal medium of sea-anemone tissues. It was hoped to demonstrate its continuity with the mesogloal fluid by observing the diffusion of aqueous dyes after micro-injection, but this procedure has not been satisfactory. Nevertheless, since tissue fluid facilitates metabolic exchanges between neighbouring cells, in this case it would certainly promote the local integration of biochemical processes, especially in animals as large as sea-anemones, which have no circulatory system within the tissues. Diffusion could proceed readily in a fluid layer of these dimensions. There is at present insufficient knowledge of metabolic functions in actinians for a detailed discussion of the subject: but it may be pointed out that owing to its distribution the subepithelial fluid provides the immediate environment for the axons of nerve and sensory cells, of whose activities much is already known (Pantin, 1952), and for all the epithelial elements such as mucus cells and nematocysts which are found in anemone tissues together with musculo-epithelium. The cells of the nerve net actually occupy some of the subepithelial space, since they lie above the muscle field and run between the bases of the epithelial elements.

Musculo-epithelial cells occur widely among cnidarian coelenterates. They

FIG. 3 (plate). A, amoebocytes in a fairly stretched mesentery. Epithelial nuclei can just be seen. Baker's formaldehyde-calcium, chlorazol black E.

B, aggregation of amoebocytes in mesogloea of body-wall. Tangential section, with circular muscle on right. Baker's formaldehyde-calcium, Mallory.

C, part of *Calliactis* sphincter in slightly oblique cross-section, showing muscle-fibres in cylindrical bundles within the mesogloea. Frozen section after Baker's formaldehyde-calcium, stained Mallory.

D, tangential section of body-wall showing condition of the mesogloea (darkly stained) at the insertion of mesenteries. Circular muscle-fibres divide the mesogloea into strands at X, as also in fig. 4. Baker's formaldehyde-calcium, Mallory.

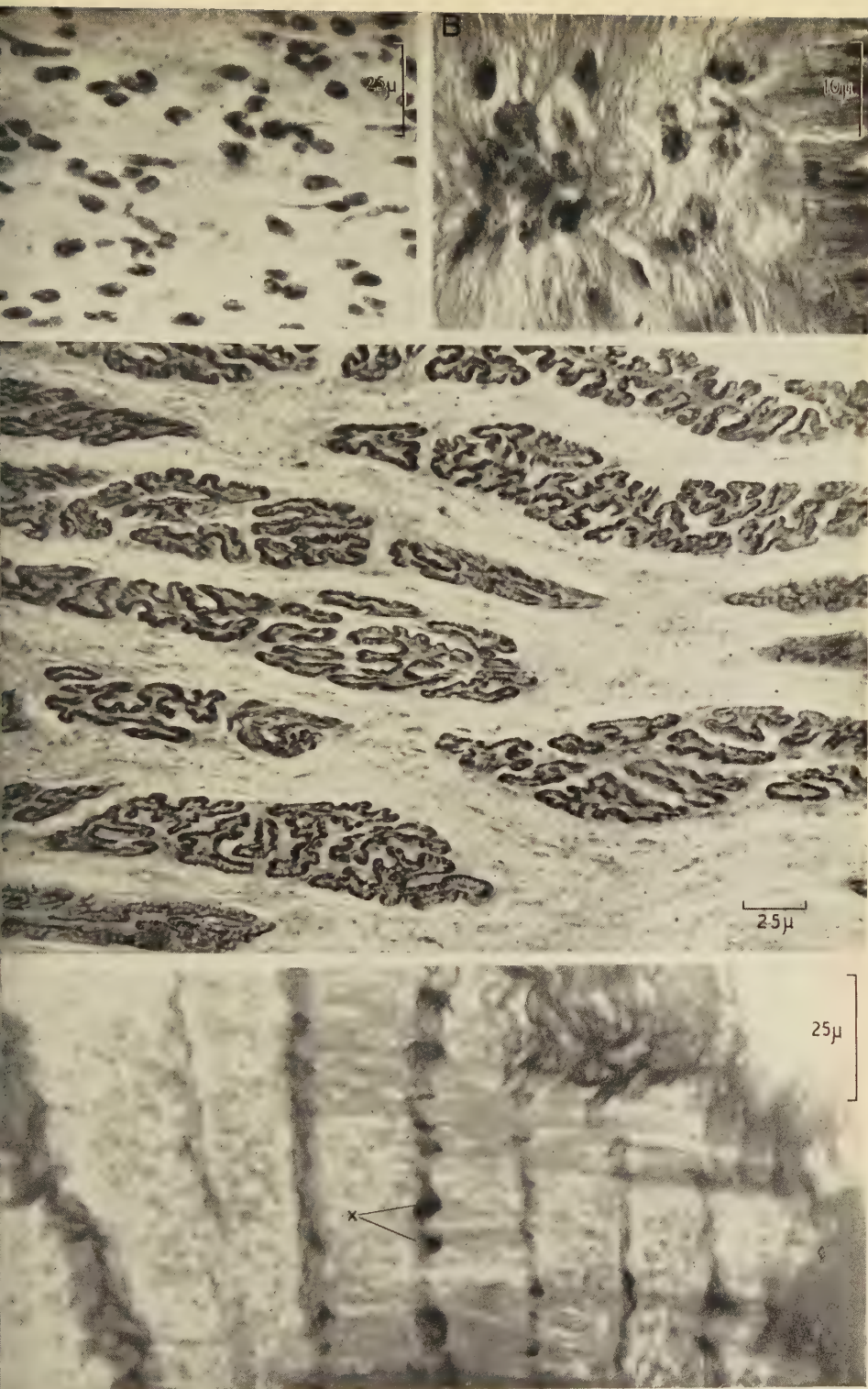


FIG. 3
E. A. ROBSON

ere first described by Kleinenberg (1872), whose discovery of the 'neuro-vascular' cells of *Hydra* was much discussed at the time. They have since been found in medusae (Krasinka, 1914), in *Lucernaria* (Korotneff, 1876), *Peritillum* (Bujor, 1901), and *Gorgonia* (Chester, 1913), and Antipatharia (Pantan, 1920) and other orders of coelenterates.

More recent studies of *Hydra* (Goodrich, 1942; Mueller, 1950; Semal van Ansen, 1952) have shown that the isolated musculo-epithelial cells resemble on a smaller scale those of *Metridium*; they differ in that the endodermal cells are phagocytic, and that in the ectoderm each cell has several muscle-fibres and lacks flagella. The muscle layer of *Hydra* has been variously interpreted as a contractile network (e.g. Mueller) and as part of the mesogloea (Holmes, 1950), but only Hadzi (1909), who was primarily interested in the nervous system, has considered the musculo-epithelium as a whole. He describes large fluid-filled vacuoles in the epithelium, which interconnect through a mesh of vertical protoplasmic strands. His figure suggests a system of sub-epithelial fluid analogous to that in *Metridium*. Sections of fixed *Hydra*, on the other hand, usually show much larger spaces within the cells than between them, and when they are compared to Hadzi's diagram it is clear that the whole problem needs re-investigating.

It seems likely, nevertheless, that in many coelenterates the musculo-epithelial tissues possess in some degree a layer of subepithelial fluid. It provides a mechanism which overcomes the viscosity of epithelial protoplasm, and therefore gains significance in well-developed muscles which contract rapidly, such as the mesenteric retractor of *Metridium*; and in fact, it is usually in such tissues that the subepithelial space is most evident (see also fig. 3, c). In partial or localized contractions a certain amount of shearing movement must take place, and the fluid layer, together with the elastic properties of the cells, will allow this to proceed smoothly.

The actinian muscle-field is modified in special anatomical regions, of which the sphincter is an important example in *Metridium*. The structure of the mesogloeaal sphincter of the related anemone *Calliactis parasitica* Couch is shown in fig. 3, c. It is primarily a specialization of the body-wall circular muscle, in which numerous folds of the muscle-field have sunk into the mesogloea and become pinched off as separate bundles. Each bundle forms a cylinder: the muscle-fibres line the periphery, and often undergo yet further folding. The cell-bodies of the muscle-fibres face the axis of the cylinder and are reduced in size. Their free epithelial surface has been lost in this situation, but each cylinder nevertheless contains the equivalent of a subepithelial space and is filled with a small amount of fluid. This will assist rapid contractions and it therefore retains a hydrostatic function. Nerve-cells supplying the sphincter, which have not yet been detected histologically although the evidence for them is conclusive (Pantin, 1935), may be expected to run in the subepithelial space as they do in other tissues.

The general circular muscle of the body-wall also divides into bundles wherever it passes beneath the attachment of a mesentery (Batham and Pantin,

1951), as illustrated by the model shown in fig. 4. The mesogloea of the mesentery is continuous with that of the body-wall by short strands, which interdigitate with the circular muscle bundles. The mesogloelial strands are marked X in figs. 3, D and 4. Fibres of the circular muscle, unlike those of the sphincter, are part of a true musculo-epithelium, and all the cell-bodies contribute to the epithelium covering the muscle-field (omitted in fig. 4 for the sake of clarity). The epithelia of the circular and parietal muscles meet along the junction of mesentery and body-wall, and the parietal fibres seem to abut right on to those of the circular muscle (see fig. 4). The circular muscle fibres do not

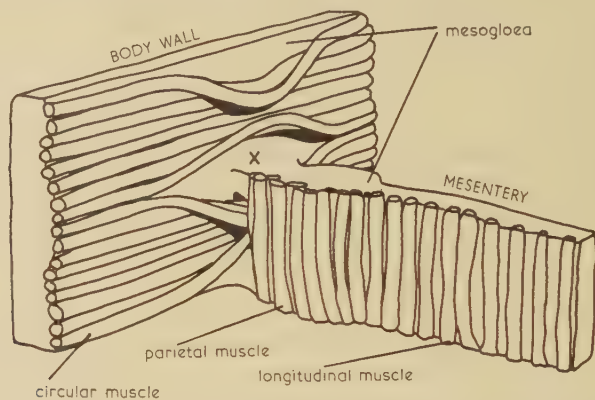


FIG. 4. Diagram showing how circular muscle of the body-wall rolls up into cylindrical bundles as it passes under the base of a mesentery. Mesogloea of body-wall and mesentery is continuous at X. Epithelial layer omitted for clarity.

appear to carry cell-bodies with them as they dive under the mesentery, but the subepithelial space continues through the cylinders and may contain strands of protoplasm. Since the nerve-net runs in the subepithelial space, it is possible to see how various regions of the circular and parietal muscles may be co-ordinated. Such co-ordination is an important feature in much of the slow, reciprocating activity characteristic of sea-anemone behaviour (Batham and Pantin, 1954), and further histological study of this region should prove rewarding.

In triploblastic animals, muscular tissue is built up in depth and does not usually arise from epithelium. There are, nevertheless, records of muscular epithelium from several groups outside the coelenterates. Among Turbellaria, *Prorhynchus* is one of several primitive though unrelated genera in which it has been reported (Hyman, 1951), but although the epidermal muscles are very near the surface, sections of *P. putealis* Haswell do not show signs of a true musculo-epithelium (fig. 5).

After staining with Mallory, sections of a specimen fixed in Bouin's fluid (kindly provided by Dr. C. F. A. Pantin) show that while several layers of epidermal muscle are present, they are separated from the outer layer of

cytoplasm by an envelope of connective tissue. As may be seen from fig. 5, beneath this 'basement membrane', the muscles form four strata of alternating circular and longitudinal fibres, the latter being most strongly developed. The structure of the outer layer of cytoplasm is not clear in this specimen, although it appears to be non-nucleate and must therefore be connected to the underlying tissues: it probably represents the peripheral part of sunken epithelial cells,

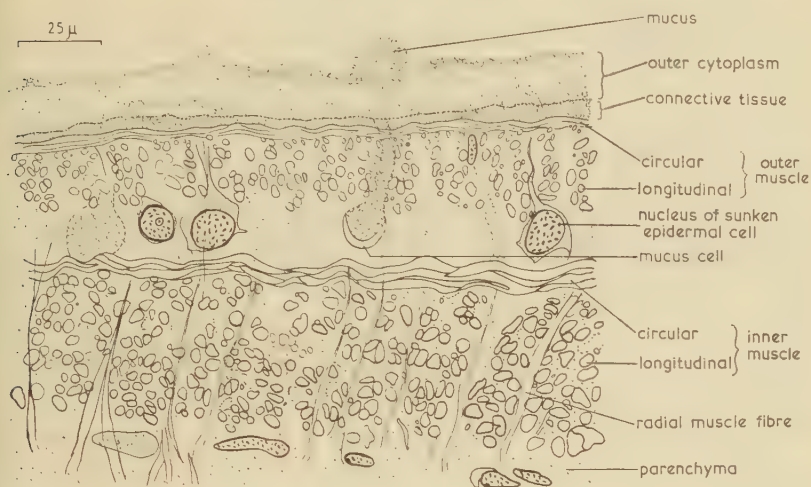


FIG. 5. Drawing of part of a transverse section of *Prorhynchus putealis* Haswell, to show disposition of surface layers.

whose nuclei are sandwiched between the two double layers of muscle. The arrangement of these four layers of muscle beneath a membrane of connective tissue is, however, such that none could form part of a true musculo-epithelium. The epidermis is nevertheless remarkably plastic in such animals, and there may be a functional parallel.

Among annelids, while certain cells lining the blood-vessels of polychaetes can contract independently (Hanson, 1951), true musculo-epithelium again seems to be absent. On the other hand, sections of the earthworm (fig. 6) and also of several polychaetes show that an analogous system may be present in the longitudinal muscle of the intestine. Here a flexible columnar epithelium is just above a single layer of muscle-fibres, resembling the ectoderm of disk and tentacles in *Metridium* in structure, and perhaps the musculo-epithelium of the endoderm in function. The blood sinuses of polychaetes must incidentally have a hydrostatic effect wherever they run near the periphery of contractile tissue. In fact, intercellular fluid probably has some mechanical importance in lower invertebrates wherever it fills in the surface layers of loosely packed tissue, and a study of several groups from this point of view would be very interesting.

Vertebrates all possess some form of myoepithelium. It has been studied particularly in sweat and mammary glands, and consists of stellate cells which

probably form a network (Richardson, 1949) and contract independently, but they do not resemble coelenterate musculo-epithelium at all. The dilator pupillae of the iris, however, which is also ectodermal in origin, retains a primitive structure in the adult (Heerfordt, 1900) which can appear very

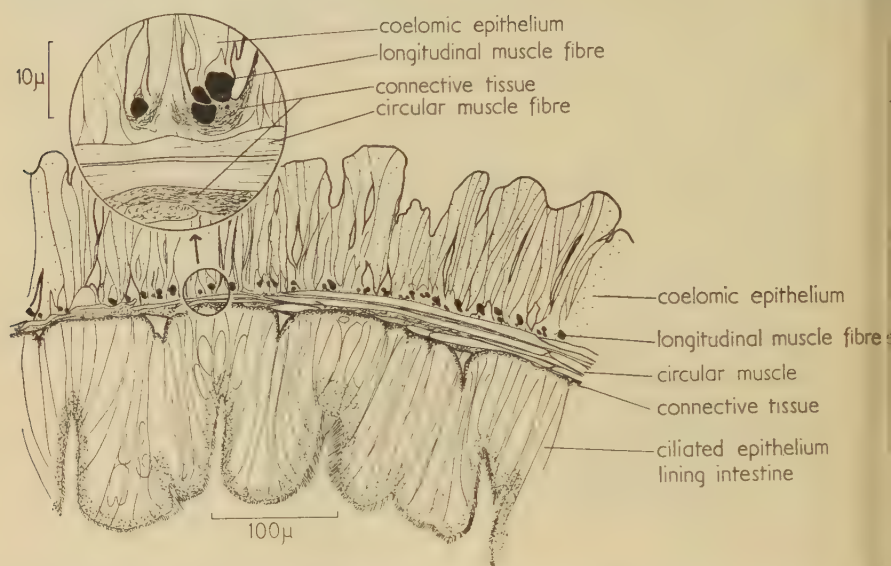


FIG. 6. Drawing of part of a transverse section of earthworm intestine to show the position of longitudinal muscle-fibres beneath the coelomic epithelium.

similar to that of certain tissues in *Metridium*. The muscle-fibres are smooth, they are attached to a compact cell-body which forms the overlying pigmented epithelium, and as in the retractor of *Metridium* (Batham and Pantin, 1951) permanent buckling seems to have occurred. It would be striking to find functional similarity in such diverse situations.

It may be seen that while true musculo-epithelium is almost entirely confined to the cnidarian coelenterates, analogous tissues may occur in higher animals wherever a flexible epithelium covers unstriated muscle. In addition the actinian muscular system resembles certain smooth muscles of higher animals in many of its physiological properties (Batham and Pantin, 1951). The excitation and propagation of contractions, however, is not fully understood in either case, and it is to be hoped that future work will throw light on such problems. The present account has emphasized some of the differences between the so-called primitive tissues of coelenterates and those of triploblastic animals: but it is likely that even special features such as the hydrostatic role of subepithelial fluid, and the supracellular organization of the muscle-field, have functional parallels among a large number of other organisms.

This research was carried out during the tenure of a D.S.I.R. assistantship under Dr. C. F. A. Pantin, F.R.S., in the Department of Zoology, Cambridge.

and at the Marine Biological Station, Plymouth. It is a pleasure to express my sincerest thanks to Dr. Pantin for his never-failing interest in the work and for much stimulating discussion and advice. I also wish to thank the Director and Staff at Plymouth for providing working facilities and a supply of living material.

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A Perfusion Chamber for Cinemicrographic Studies

By JOHN PAUL

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University of Glasgow)

SUMMARY

A perfusion chamber is described which eliminates most of the difficulties encountered with other chambers.

MOST perfusion chambers hitherto described for time-lapse cinemicrographic studies of living cells have defects. Chambers of the type originally described by Pomerat (1953) provide good optical conditions and shallow chamber but require some skill to manage and, being sealed with wax, tend to leak unexpectedly. This difficulty is overcome by the designs of Rose (1954) and Richter (1955) in which the chamber is formed by sand-

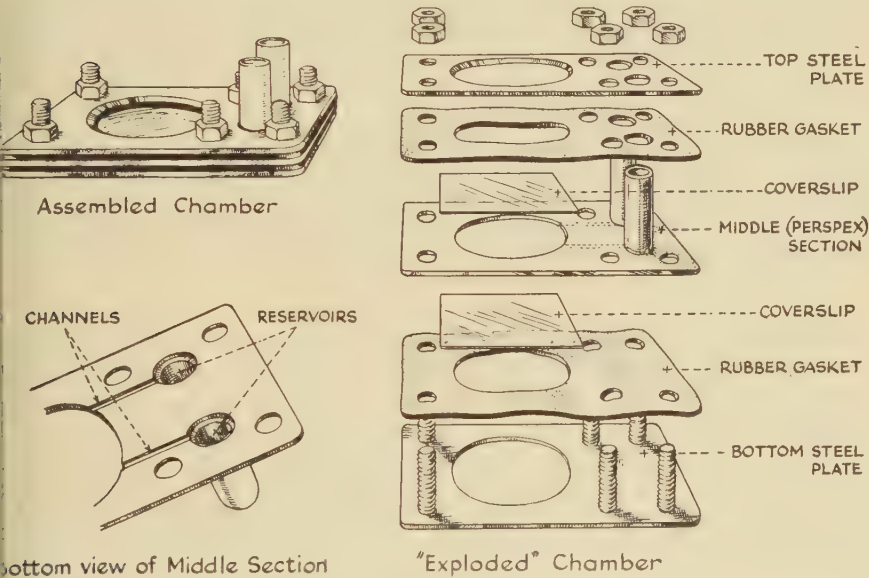


FIG. 1.

wiching a rubber gasket between metal plates, but in these cases it is usually too deep to permit accurate focusing of the substage condenser at high magnifications.

The chamber described here is a compromise based on the above designs and an ingenious one proposed by Dick (1955). It can be made easily in a day and so in any workshop and in use it can be assembled in 5 min. with the minimum of skill. The construction is clear from the diagram. Dimensions can

be adjusted to suit requirements. Our own chambers are 3×2 in. (77×51 mm) with a chamber $1\frac{1}{4}$ in. (32 mm) in diameter for use with $1\frac{1}{2}$ in. (38 mm) square no. 1 coverslips. The channels running along the bottom of the perspective section act as ducts from the reservoirs to the chamber when all parts are clamped together, the bottoms of the canals and reservoirs being formed by the lower rubber gasket. The depth of our own chambers is 1.5 mm and this is capable of further reduction.

When the apparatus has been assembled it is filled by placing medium in one of the reservoirs and tilting so that all the air (if desired) is removed before the medium flows through into the second reservoir. To change the medium, fluid is added to the filling reservoir and withdrawn from the emptying reservoir. This operation can be performed by means of a Pasteur pipette inserted through a $\frac{1}{4}$ -in. hole in the top of the microscope incubator which does not therefore have to be opened. When properly made and assembled the chamber cannot leak. A further advantage is that the well formed above the top coverslip can be filled with water for use with water immersion objectives of the type used in interference microscopes.

I wish to thank Mr. Ian McLardy for his technical skill in making the prototypes to my design.

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The Shrinkage of Air-dried Bacteria prepared for the Electron-microscope

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AND K. DEUTSCH

(Department of Zoology, University of Edinburgh)

With one plate (fig. 1)

SUMMARY

Values for the mean thickness of living bacilli in a number of different cultures of *Actobacillus bulgaricus* were obtained from phase-change measurements. These were made with an interference microscope. The bacilli were mounted in media of several different refractive indices. The accuracy of these measurements is not limited by the wavelength of the light used, so that the mean thickness of bacteria can be measured more critically in this way than by an eyepiece micrometer scale.

The values obtained for the mean thickness of the living bacilli were compared with the mean width of similar bacilli measured in electron-micrographs, after they had been prepared for electron-microscopy by drying them in air on formvar. The method used for preparing the bacteria for the electron-microscope is described in detail.

It was found that osmium-fixed air-dried *L. bulgaricus* so prepared shrink linearly to the plane of the formvar film by $59\% \pm 5\%$, and similar unfixed bacilli by $51\% \pm 5\%$. Phase-change measurements through these air-dried bacilli indicated a similar degree of shrinkage, or a slightly greater shrinkage, in the direction at right angles to the formvar film.

INTRODUCTION

THE high resolution and accurate calibration of modern electron-microscopes enables the dimensions of objects of the size of bacteria to be measured with considerable accuracy; but, in the case of bacteria, the necessity of placing the material in a high vacuum for examination by electron-microscopy means that the water they originally contained has to be entirely removed, so that it is certain that there must be a considerable loss of volume. So far, no attempts have been made to estimate the shrinkage of bacteria so prepared owing to the difficulty of making accurate measurements of the size of living bacteria. Such measurements, at present, can only be made with a microscope using visible light (or the relatively non-lethal wavelengths of the far ultra-violet), and they are usually made with an eyepiece-micrometer scale in an ordinary light microscope. Accuracy is here limited by the numerical aperture of the objective and the wavelength of light used, and it is scarcely possible to determine the dimensions of an object in this way more accurately than to the nearest 0.4μ ; which means that, when objects of the size of living bacteria are measured, the error may be considerable.

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An interference microscope, however, enables such measurements to be made with visible light in a manner which is not subject to these particular limitations, and which appears to be appreciably more accurate. With an interference microscope the retardation in phase of light passing through a small object, such as a living bacterium, can be measured very critically. This phase-change is proportional to the product of the refractive index of the bacterium relative to that of the mounting medium, and its thickness. The thickness of bacteria can therefore be deduced from phase-change measurements of this kind if the refractive indices of the mounting medium and of the organism are known; or, if the refractive index of the organism is not known, both its thickness and refractive index may be found by making similar phase-change measurements in two mounting media of different refractive indices.

Both these methods have recently been used to obtain values for the mean thickness of a living bacterium, *L. bulgaricus* (Ross 1955, and to be published); and it was decided to measure the same organism with an electron-microscope in order to estimate the amount by which these bacilli shrink.

MATERIAL

The organism measured, a typical strain of *L. bulgaricus*, was obtained in milk cultures supplied by a commercial yoghurt firm (Les Laboratoires 'Yalacta', 51 Rue Lepic, Paris), where it is grown under very constant conditions. The living bacilli (which are rod-shaped), always appeared to be between 1μ and 1.5μ in diameter; and eyepiece-micrometer measurements showed no detectable variations in the thickness of the bacilli in cultures supplied at different dates or in the sub-cultures grown from them in sterilized milk. In addition to this one strain of bacilli, the cultures contained small numbers of two other organisms that were easily distinguishable, a strain of *Streptococcus* and an encapsulated *Diplococcus* (fig. 1, A-D); but only the diameters of the *L. bulgaricus* were measured.

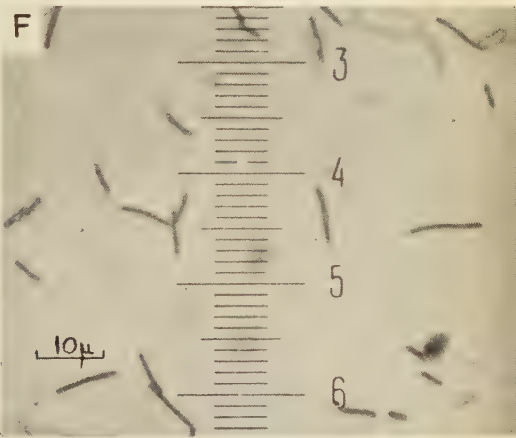
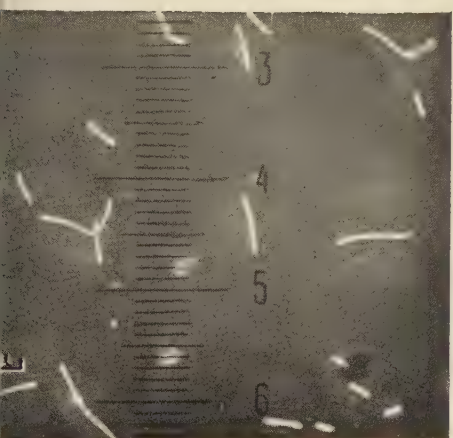
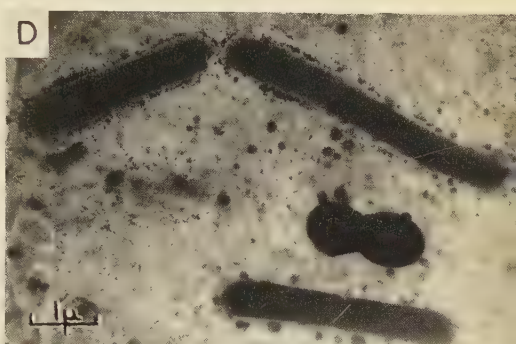
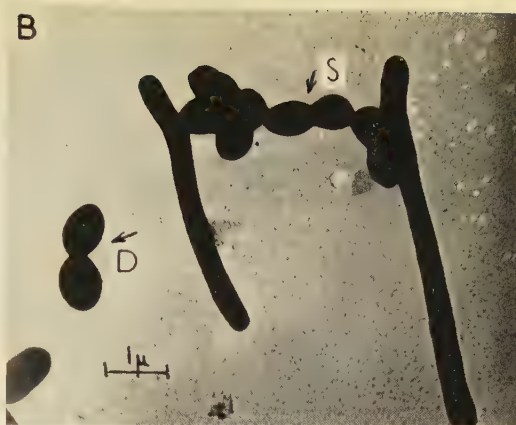
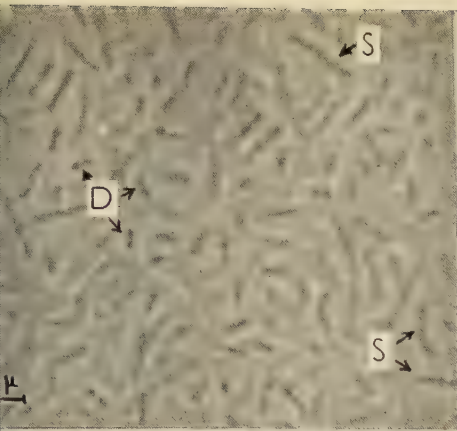
FIG. 1 (plate). A, living bacteria from a yoghurt culture, mounted in 0.2% sodium chloride, photographed with a 2-mm fluorite phase-contrast objective with a 25% absorbing positive phase plate. The bacillus *Lactobacillus bulgaricus* predominates, but a species of *Streptococcus* (S) and an encapsulated *Diplococcus* species (D) can also be seen.

B and C, electron-micrographs of similar yoghurt bacteria to those in A, after being fixed with osmium tetroxide and air-dried on a formvar film.

D, electron-micrograph of similar yoghurt bacteria after being air-dried on formvar without fixation. A pale surface deposit round each organism is clearly visible.

E, similar yoghurt bacteria fixed with osmium tetroxide and air-dried on a glass slide, photographed in air with a Smith interference microscope with a 2-mm double-focus objective and an Ilford 807 mercury-green filter. The analyser of the interference microscope has been set at 140° , which gave a maximally dark field.

F, the same preparation of bacteria in air as in E, with the analyser of the interference microscope set at 73° . At this setting, some of the bacilli in the field appear maximally dark. (The difference between this analyser setting and that in E, represents a phase retardation (in air of 134°).



K. F. A. ROSS and K. DEUTSCH

METHODS AND MEASUREMENTS

The methods of measuring the living bacilli

The two methods used for measuring the mean thickness of the living bacilli will only be described briefly, because they will be fully reported and discussed elsewhere.

(1) With the first method, the mean refractive index of the bacilli was obtained by direct measurement by the method of immersion refractometry first employed by Barer and Ross in 1952, which is fully described by Barer and Joseph (1954, 1955*a*). The bacilli were mounted in a series of protein solutions of different concentrations, until one was found in which the majority of the bacilli showed up with minimum contrast when examined with a phase-contrast microscope. The refractive index of this solution (in which approximately equal numbers of bacilli appeared slightly darker and slightly brighter than the medium) was 1.4045 in all the cultures measured; and this was taken as the value of the mean refractive index of the *L. bulgaricus*.

All the refractive index measurements were made at room temperature with a Bellingham & Stanley pocket refractometer. This instrument has a built-in yellow filter with a transmission spectrum equivalent to the mean of the two sodium lines (589 mμ); and, with it, measurements could be made accurately to the nearest 0.001, or more accurately than this.

The bacilli were then mounted in dilute saline (0.25% NaCl), and a Smith interference microscope (manufactured by Messrs. Charles Baker of Holborn) was used to measure the retardation of light passing through bacilli lying with their long axes at right angles to the optical axis of the microscope. Phase-change measurements were made through ten such bacilli in each culture, and the mean of these measurements found. A value for the mean thickness in microns of the population in each culture *t* was then calculated from the formula

$$t = \frac{\phi}{360} \times \frac{\lambda}{n-m}, \quad (1)$$

where ϕ = the mean phase retardation measured through the bacilli expressed as an angle, n = the mean refractive index of the bacilli (1.4045), m = the mean refractive index of the saline mounting medium (1.3350), and λ = the mean wavelength of the light used (0.542 μ, obtained by using a tungsten 'pointolite' lamp with an Ilford 807, mercury green, gelatine filter).

This method has already been described by Ross (1955), except that, in that account, a small correction factor was used ($\times 1.13$ or $\frac{2}{\sqrt{\pi}}$), and further investigations have shown that this was probably not necessary. Measurements were made on 14 different cultures by this method. The values obtained for the mean thickness of the living bacilli in each culture ranged from 1.13 μ to 1.23 μ and the mean of all the mean values obtained by this method was 1.16 μ.

(2) In the second method used, similar phase retardation measurements were made through the bacilli mounted in saline, and also through bacilli

from the same cultures mounted in protein solutions with various refractive indices between that of saline and the organism. These two phase-change values were then used to calculate the mean thickness t of the bacilli in each culture from the formula

$$t = \frac{\phi_1 - \phi_2}{(m_2 - m_1)360} \times \lambda, \quad (2)$$

where ϕ_1 = the mean phase retardation through the bacilli in saline and ϕ_2 = the mean retardation through the bacilli in the protein solution, expressed as angles, m_1 = the refractive index of the saline solution, m_2 = the refractive index of the protein solution, and λ = the mean wavelength of the light used (0.542μ , as before).

This method was similar to that employed by Barer in 1953 for measuring the thickness of mouth epithelial cells, except that, instead of the phase-change measurements being made on the same individual cells suspended successively in the two mounting media, they were made on a different sample of the bacterial culture in each medium, as it was not possible to keep the same bacterium in the field and change the mounting medium. The method is fully described by Ross and Billing (1957), who have used it for measuring bacterial spores.

Measurements were made on 9 different cultures by this method, and the values obtained for the mean thickness of the bacilli in each culture ranged from 1.02μ to 1.14μ ; and so they were slightly lower than the values obtained by the previous method. The average of all the values obtained was 1.09μ .

The method of preparing the bacilli for electron-microscopy

There is no generally accepted technique for preparing dried films of bacteria for the electron-microscope. The methods used here will be described in detail.

The most important thing was to ensure that the suspension fluid in which the organisms were finally evaporated on the formvar film of the specimen grid was as free as possible from dissolved solids, so that no appreciable deposit was left round the bacteria that could obscure their outline or make them appear thicker than they really were. The original milk-foam culture media in which the organisms were supplied contained many visible solid particles and fat droplets as well as proteins and sugars in solution. All these had to be separated from the bacteria by centrifuging and washing.

The method used, which was evolved and found satisfactory after a certain amount of trial and error, was as follows:

(i) The original culture was centrifuged at a slow speed, with the result that it separated into a small precipitate of dense solid matter, and an upper fraction of milky froth. Three-quarters of this upper fraction was then removed with a pipette and thoroughly mixed with one-third of its volume of distilled water. The mixture was then recentrifuged at slow speed. This precipitated most of the remaining extraneous solid matter and left a pale grey

supernatant fluid, which, on examination, was found to be rich in suspended bacteria.

(ii) To 1 ml of this supernatant fluid, 1 ml of 1% aqueous osmium tetroxide solution was added and thoroughly mixed. This mixture was then left to stand for 2 h., after which time practically all the remaining extraneous matter had separated out as a black precipitate.

(iii) 1 ml of the surface liquid was then removed and mixed with 5 ml of distilled water and centrifuged at high speed. The bacteria formed a small solid precipitate. All but about 0.1 ml of the supernatant fluid was then removed and the precipitate of bacteria was mixed again with the remaining fluid; and 5 more ml of distilled water were added. This mixture was again centrifuged at high speed, and again all but about 0.1 ml of the supernatant fluid was removed, and the bacterial precipitate remixed with the remaining fluid.

(iv) Drops of this suspension were applied directly to the formvar films on the electron-microscope specimen grids with a fine-drawn pipette, and left to evaporate at room temperature.

The osmium-fixed *L. bulgaricus* prepared in this way appeared in electron-micrographs with sharp and regular outlines. They showed no appreciable deposit on their surface, and there was very little extraneous matter apparent in the rest of the field. The *Diplococcus* species usually appeared without its surrounding capsule (fig. 1, B, C).

In addition to the fixed bacteria some preparations of unfixed bacteria were also made by exactly the method described above, with the omission of stage (ii). In these, however, the bacteria in the electron-micrographs frequently appeared to be surrounded by a distinct pale zone which was probably a deposit; and, on this, appreciable numbers of denser particles could usually be seen (fig. 1, D). Narrow pale zones at the edges of dried bacteria appear quite frequently in published electron-micrographs (e.g. in those of *Bacillus megaterium* by Dubin and Sharp, 1944); and it is probable that they usually represent surface deposits from the media.

None of the air-dried bacteria on the formvar films were gold-shadowed or subjected to any other process involving the deliberate addition of surface deposits. This was not necessary since the outlines of the unshadowed bacteria appeared sharp in all the electron-micrographs.

The methods of measuring the bacilli prepared for electron-microscopy

The mean thicknesses of the living bacilli were deduced from phase-change measurements made in one dimension only—the direction of the optical axis of the interference microscope. It was assumed that the living bacilli were circular in cross-section, and that their thickness in this direction was equal to their width in the plane at right angles to it.

Such an assumption, however, was not justified in the case of the air-dried bacilli prepared for the electron-microscope, because it seemed not unlikely

that they might become flattened in the process of drying, and so might shrink less in the plane of the formvar film than in the plane at right angles to it. Consequently it was desirable to measure their dimensions in both planes if possible.

(1) The *widths* of the air-dried bacilli in the plane of the formvar film were measured directly from electron-micrograph prints.

The electron-micrographs were taken with a Siemens Elmiskop I at 80 kV and an instrumental magnification of $\times 8000$. The magnification was calibrated according to a method described by the makers, i.e. objective, intermediate, and projector lens (first pole-piece) are switched on and the current in the projector lens is adjusted until the circular hole of 70- μ objective aperture forms an image on the screen with a diameter of 90 mm. The accuracy of the calibration is $\pm 3\%$, which was adequate for our measurements.

In all, a total of 88 osmium-fixed bacilli and 33 unfixed bacilli were photographed and measured. All the measurements were made at an arbitrarily chosen point 1μ from one end of each bacillus, because the width of individual bacilli in the electron-micrographs frequently varied slightly along their length. Similar measurements were made on the original photographic plates because printing paper sometimes shrinks appreciably after being washed and dried (Pusey, 1956), but these did not differ significantly from the print measurements.

The widths of the osmium-fixed bacilli thus measured ranged from 0.375μ to 0.560μ , with a mean of 0.475μ ; and those of the unfixed bacilli ranged from 0.440μ to 0.625μ , with a mean of 0.545μ .

(2) The mean *thickness* of the air-dried bacilli in the other plane, i.e. at right angles to the plane of the formvar film or in the direction of the axis of the electron beam, was measured with the interference microscope by the second of the two methods already described for measuring the living bacilli.

This was done with a sample of the final suspension of the osmium-fixed bacilli, prepared in the manner described above and evaporated on an ordinary glass slide. Phase-change measurements were made through 10 of these dried bacilli in air, and then the preparation was covered with distilled water, and the phase-changes through 10 similar bacilli were measured. The mean thickness of the bacilli t was calculated from formula (2) above (page 284), where ϕ_1 = the mean phase retardation through the bacilli in air, and ϕ_2 = the mean phase retardation through the bacilli in water, expressed as angles, n_1 = the refractive index of air (1.0), n_2 = the refractive index of water (1.334), and λ = the mean wavelength of the light used (0.542μ , as before). It was assumed that the bacilli did not swell appreciably when they were re-immersed in water after they were dried. The bacilli were measured on a glass slide rather than on formvar film because the latter was found to be too uneven in thickness to provide an adequate reference field for the interference microscope. It was thought probable that, if any appreciable flattening of the bacilli did occur, this would be even more apparent on a rigid glass surface.

The mean phase-change through the osmium-fixed bacilli in air was

found to be 139.2° , and in water 41.2° (fig. 1, E, F). This gave a value of 0.445μ for their mean thickness in this direction, which is very closely comparable to the value of 0.475μ obtained from the electron-micrographs for their mean width in the opposite direction. Thus it would appear that the osmium-fixed bacilli do not flatten or spread to any significant extent when they are dried on formvar films, but shrink uniformly and maintain their cylindrical cross-section.

It should be pointed out, however, that the validity of this conclusion depends on the assumption that the air-dried bacilli do not swell again appreciably when they are covered with distilled water for the second phase-change measurement. If this is not true, and the bacilli do swell, one would expect their mean phase retardation in water (ϕ_2) to be rather lower than if no swelling occurred: and this would give rather higher values for their mean thickness than was actually the case. Thus the very close correspondence between the values for the mean width obtained by electron-microscopy and the mean thickness obtained by interferometry do necessarily mean that there has been no flattening. The mean refractive index of the air-dried bacilli calculated from the phase retardation measurements was 1.470. This is rather lower than the values of 1.53–1.54 that have been obtained for the refractive indices of dried protein products such as leather and dried casein (Chamot and Mason, 1938); and it consequently indicates that the thickness measurements obtained in this way are probably slightly too high, and the bacilli are, in fact, slightly flattened.

Thus, all that can be said with certainty is that the shrinkage of bacilli dried on a glass surface in the direction of the optical axis of the microscope is *at least* as great as the shrinkage in the plane at right angles to it; and it may be even greater. A more uniform shrinkage might reasonably be expected in the bacilli dried on the formvar films, since, unlike the glass, the formvar is pliable.

It is probable that the same arguments apply in the case of the unfixed bacilli, but only the osmium-fixed bacilli were measured in this way.

RESULTS

Linear shrinkage

The mean linear shrinkage in diameter of the bacilli s is given by the formula

$$s = 1 - \frac{t_2}{t_1}, \quad (3)$$

where t_1 = the mean thickness of the living bacilli estimated by each of the two methods used, and t_2 = the mean thickness of the air-dried bacilli, both in the plane of the formvar film from the electron-micrographs and at right angles to this from interferometry.

Table 1 shows the mean shrinkage (expressed as percentages) obtained for both the osmium-fixed and the unfixed bacilli, on the assumption, first, that the mean thickness of the living bacilli was 1.16μ (obtained by the first

method of measuring the living bacilli), and secondly, that it was 1.09μ (obtained by the second method).

It will be seen that the mean shrinkage values so calculated differ from each other by a maximum of 3%, and that, in the case of the osmium-fixed bacilli, the shrinkages in one plane differ from those in the other plane by a maximum of 4%. The shrinkage values for the unfixed bacilli are about 10% lower, and differ from each other by 3%.

The accuracy of the shrinkage values

The accuracy of the above values for linear shrinkage in diameter depends on the accuracy of the values obtained for the mean thickness of the living bacilli and the accuracy of the measurements on the electron-microscope preparations.

TABLE I

The mean thickness of living and air-dried Lactobacillus bulgaricus measured by interferometry and from electron-micrographs, and the linear shrinkage of the air-dried bacilli calculated therefrom

<i>Mean thickness of living bacilli (t_1)</i>	<i>Mean thickness or width of air-dried bacilli (t_2)</i>	<i>Linear shrinkage of the air-dried bacilli (s)</i>
<i>Osmium-fixed bacilli</i>		
(a) Width of air-dried bacilli measured from electron-micrographs		
1.16 μ method (1)	0.475 μ	58%
1.09 μ method (2)	0.475 μ	56%
(b) Depth of air-dried bacilli (along the optical axis) measured by interferometry		
1.16 μ method (1)	0.445 μ	62%
1.09 μ method (2)	0.445 μ	59%
<i>Unfixed bacilli</i>		
Width of air-dried bacilli measured from electron-micrographs		
1.16 μ method (1)	0.545 μ	53%
1.09 μ method (2)	0.545 μ	50%

The calibration of the Siemens electron-microscope used is such that the error in measuring the electron-micrographs is less than $\pm 3\%$; so the main source of error lies in the estimations on the living material. For reasons that will be fully discussed elsewhere (Ross, 1957), it is thought unlikely that the error in the measurements made of the mean thickness of the living bacilli is more than $\pm 0.1 \mu$. Consequently, it is very unlikely that the error in the values obtained for the mean shrinkage of the bacilli prepared for electron-microscopy is greater than $\pm 5\%$.

Thus, linear shrinkage values of $59\% \pm 5\%$ for the osmium-fixed bacilli and $51\% \pm 5\%$ for the unfixed bacilli are unlikely to be far wrong.

DISCUSSION

A linear shrinkage of between 50% and 60% is very considerable: but it is not entirely surprising in view of the fact that the material has been completely desiccated.

The shrinkage is almost twice as great as that found in ordinary tissue-cells after fixation and paraffin-wax embedding (Ross, 1953); and it is probable that bacteria and other cytological material prepared for electron-microscopy by embedding (e.g. in *n*-butyl methacrylate) may shrink less than this 50–60%. Even so, under certain circumstances they may shrink quite considerably, and the consequent reduction and distortion of intracellular spaces may contribute to the difficulty of interpreting cytological electron-micrographs.

These shrinkage values were only obtained for a single species of bacillus, *Lactobacillus bulgaricus*; but it is likely that many other species of bacteria may similarly show considerable shrinkages when they are dried in air. This is because refractive index measurements indicate that many species of bacteria appear to contain rather similar concentrations of solid matter when they are alive. The *L. bulgaricus* vegetative cells used here have a refractive index of about 1.4045, which is equivalent to about 40% cell solids (Ross, 1955), and this is similar to that found in *Streptococcus haemolyticus* (Barer, Ross, and Kaczyk, 1953). The vegetative cells of *Bacillus cereus*, *B. cereus* var. *mycoides*, and *B. megaterium* all have rather lower refractive indices between 1.3830 and 1.4030, equivalent to cell-solid concentrations of 27–38% (Ross and Billing, 1957); and thus they might be expected to shrink rather more than *L. bulgaricus*.

The outlines of the bacilli in the electron-micrographs always appeared nearly straight and without wrinkles. This suggests that, during air-drying, the cell membrane must shrink to the same extent as the contents; and consequently it must be very elastic.

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The Cytoplasmic Inclusions of the Neurones of *Patella vulgata*

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SUMMARY

1. The cytoplasmic inclusions of the neurones of the pedal ganglia are filamentous mitochondria and lipid globules.
2. Two kinds of lipid globules occur. One is yellow, the other devoid of natural colour.
3. The yellow globules owe their colour to the presence of carotenoid. They are chemically complex, since they contain not only cerebroside and phospholipid, but also a certain amount of amino-acids (presumably as protein) and carbohydrate.
4. The other globules consist almost entirely of cerebroside and phospholipid.
5. Four kinds of neurones are present, of which three are unipolar and one bipolar. The three kinds of unipolar neurones are easily distinguishable by their morphological characters and by their differing content of lipid globules.

THE cytoplasmic inclusions of the neurones of *Patella vulgata* have been studied by Lacy and Rogers (1956) and Lacy and Horne (1956). These authors studied thin sections under the electron microscope and made 'Golgi' preparations by Kolatchev's technique; they also examined living cells dyed with neutral red. It appeared to me desirable to check and extend their observations in several ways. I decided to undertake a much fuller study of the living cell by phase-contrast microscopy and the use of a wide range of vital dyes. It was also necessary to determine the composition of the cytoplasmic inclusions by cytochemical tests.

MATERIAL AND METHODS

Living specimens of *P. vulgata* were obtained from Plymouth. The pedal ganglia ('nerve-cords') were dissected out in sea-water. Neurones are obtainable from any part of the long ganglia. Occasionally the cerebral or pleural ganglia were used instead of the pedal, but their neurones were not fully investigated. For studies by phase-contrast microscopy the ganglia were teased in sea-water, slightly compressed by the coverslip, and examined without further treatment. Positive phase-contrast was used. The following vital dyes were tried: neutral red, Janus green, brilliant cresyl blue, methylene blue, Nile blue, and dahlia. Each dye was dissolved at 0.5% in distilled water. Two drops of this stock solution were added to 2.5 ml. of sea-water, and the ganglion was gently teased in this. Dyeing was allowed to proceed for 10 to 20 min, and the fragments of tissue were then placed on a slide in the same fluid and slightly compressed by the coverslip.

The histochemical tests applied are listed in the Appendix (p. 299).

The Golgi techniques of Aoyama (1929), Weigl (Mann-Kopsch) (1910) and Kolatchev (1916) were used. Routine microscopical preparations were made.

RESULTS

The kinds of cytoplasmic inclusions

The cytoplasmic inclusions seen in neurones of *P. vulgata* are mitochondria and two kinds of lipid globules.

The mitochondria are mostly filamentous or rod-shaped. They are seen by phase-contrast microscopy. The best fixatives are Altmann, Champy and Helly; the best methods of staining are those of Metzner (Metzner and Krause, 1928) and Hirschler (1927). They appear thicker by Hirschler's than by Metzner's technique. Some of them can be blackened by the Kolatchev technique.

The two kinds of lipid globules are readily distinguished in life, one kind being yellow, the other devoid of colour.

The yellow globules vary in diameter from about $\frac{3}{4}\mu$ to $1\frac{1}{2}\mu$. They are generally not quite spherical, especially the bigger ones.

The yellow globules are vitally coloured red by neutral red; they are coloured green by methylene blue, brilliant cresyl blue, and Nile blue, by mixture of their own colour with that of the dye.

The yellow pigment is evenly distributed throughout the globules. On treatment with concentrated sulphuric acid the globules become blue-black. Their colour is therefore presumably due to a carotenoid pigment. Such pigments occur commonly in globules contained in the neurones of gastropods (Cain, 1948; Chou, 1957).

Cerebroside is probably present in large quantity in the yellow globules; since in sections of ganglia fixed in cold acetone they give a strongly positive reaction with Sudan black. No cholesterol or fatty acid has been demonstrated in them; the response to Cain's test for plasmalogen (Cain, 1948) is weak and uncertain. Phospholipid appears to be restricted to their surfaces, but this may not have been its distribution during life.

Although the yellow globules are essentially lipid, yet there are other constituents. They react quite strongly to the PAS test, but feebly after the action of saliva. This is suggestive of the presence of carbohydrate. The residual colour after the use of saliva is presumably due to the presence of cerebroside.

The globules appear to contain tyrosine and histidine. The evidence for the presence of the latter is a positive reaction to the coupled tetrazonium test controlled by blocking reactions (see Appendix). It is therefore to be presumed that the globules contain protein. They are strongly basiphil, and become orange with pyronine/methyl green (PMG); Feulgen's test is negative.

There is a feebly positive reaction to Gomori's test for alkaline phosphatase

The globules that are without natural colour are easily dyed blue in life by methylene blue, and they will therefore be called 'blue' globules; neutral red will also colour them. They are spherical and generally less than $1\ \mu$ in diameter; most of them are smaller than the smaller yellow globules. Although most of them are coloured blue in life by brilliant cresyl blue, the smallest of them show the metachromatic colour of this dye. The globules are easily seen in life by phase-contrast microscopy. They appear to have a dark rim when examined by positive phase-contrast; this is presumably an optical artifact ('optical membrane' of Oettlé, 1950).

Unlike the yellow globules, the 'blue' ones are almost entirely lipid in composition. Carotenoid is absent; the two chief lipids are cerebroside and phospholipid. The former constituent is particularly easily shown by the method of Casselman and Baker (1955). Uncertain or feebly positive reactions are given to the PAS test and to the tests for amino-acids.

Both the yellow and 'blue' globules become grey with the Weigl technique, but the 'blue' ones become darker than the yellow. The Kolatchev technique is unreliable with these globules: sometimes it darkens some of them. Aoyama's method does not darken them. None of the 'Golgi' methods shows a typical 'dictyosome' picture. The 'blue' globules are coloured red by Metzner's method for mitochondria (as lipid globules often are). When a fixative containing osmium tetroxide is used, the grey caused by the osmium mingles with the red of the Metzner dye and darkens it.

The kinds of neurones

Four kinds of cells are found in the pedal ganglia. Three of these, which I shall call large, small, and yellow, are unipolar; there are also a few bipolar cells.

Large cells (figs. 1, A and 2, A). Although these are larger than the others, yet they are much smaller than the neurones of many molluscs. Their diameter, transverse to the direction of the axon, is about $13\ \mu$. The cell is pear-shaped, tapering gradually to the axon.

The yellow globules of various sizes are distributed in the periphery of the cell. The 'blue' globules are almost confined to the centre.

Small cells (figs. 1, B and 2, B). The shape is similar to that of the large cells. The diameter transverse to the direction of the axon is about $9\ \mu$. These cells are chiefly distinguished by the absence or rarity of yellow globules. The mitochondria are similar to those of the large cells.

Yellow cells (figs. 1, C and 2, C). These are markedly different from the large and small cells. They are nearly spherical instead of pear-shaped. The axon is difficult to see in the living cell unless it happens to lie at right angles to the optical axis of the microscope; but it can be seen in certain fixed preparations (for instance, in ganglia treated by Holmes's method), and there can be no doubt that these are nerve-cells. Their most striking feature is that they are almost completely filled with yellow globules. The latter resemble those in the large cell in all respects except that there are hardly any small ones: very few of them are less than $1\ \mu$ in diameter. The globules are so numerous that

the nucleus cannot be distinguished in the living cell (fig. 1, c), but it is easily seen in fixed preparations. It is remarkably small (fig. 2, c).

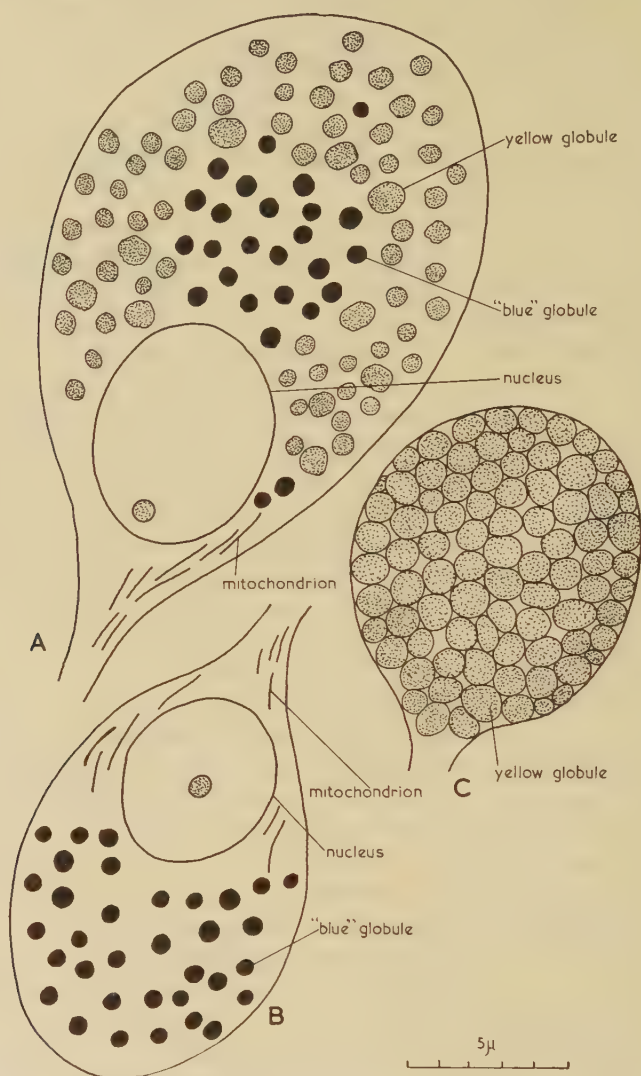


FIG. 1. Diagrams of the neurones of *Patella vulgata* as seen in life. A, large cell; B, small cell; C, yellow cell

The dense packing of the yellow globules in these cells makes the study of mitochondria difficult. In preparations fixed for mitochondria (Altman or Helly with postchroming) and dyed by Metzner's method, small mitochondria are seen between the yellow ones (fig. 2, c).

Bipolar cell (fig. 2, d). These are too infrequent for full study and I have seldom been able to distinguish them in life. The mitochondria seen in fixed

preparations are shorter than in the large and small cells, and not numerous. They are best shown by Hirschler's method. The rather scanty evidence

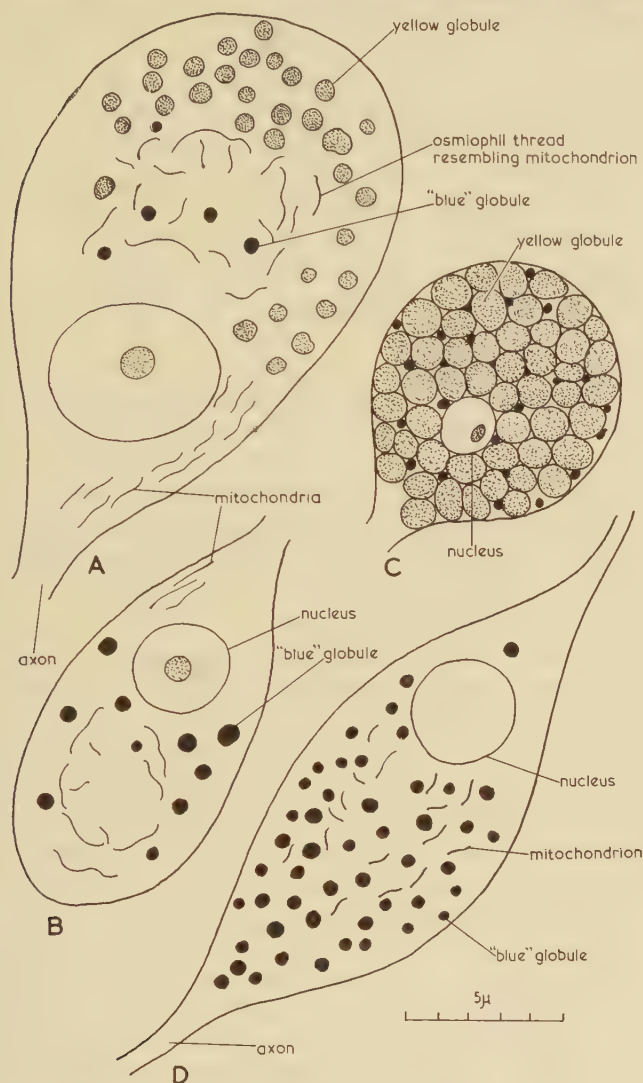


FIG. 2. Diagrams of the neurons of *Patella vulgata* as seen in fixed preparations. A, large cell; B, small cell; C, yellow cell; D, bipolar cell

suggests that the globules are of the same nature as the 'blue' globules of the large and small cells, but they are smaller.

DISCUSSION

The neurons of *Helix* are not so diverse as those of *Patella*. In the former animal I have not seen cells corresponding to the yellow and bipolar neurones of the limpet. The neurons of *Helix* are variable in size. Since the larger ones

contain yellow globules while the smaller contain few or none, there is general resemblance to the large and small cells of *Patella*, but there are no cells of intermediate size in *Patella*.

The yellow globules of *Patella* show a general similarity to those of *Helix*. They are in both cases histochemically complex, since the evidence suggests that they contain lipid, protein, and carbohydrate. The lipids of the globules are, however, simpler in *Patella*: I have only been able to demonstrate carotenoid, phospholipid, and cerebroside (the latter in great quantity). The yellow globules of *Helix* contain also cholesterol and its esters, and plasmalogens; there is much less cerebroside than in *Patella*.

The 'blue' globules of both animals contain phospholipid, but little or no protein or carbohydrate. Those of *Patella* contain cerebroside in addition to phospholipid.

It may be recollected that the yellow and 'blue' globules of *Limnaea* are similar to those of *Helix* (Chou, 1957). Thus the globule system of these three gastropods is essentially similar. The highly refractile 'colourless' globules of the neurones of *Helix* are, however, not represented in *Patella*.

All the cytoplasmic inclusions seen in the various neurones of *Helix*, *Limnaea*, and *Patella* are represented diagrammatically in fig. 3. No neurone of these three animals actually contains all the kinds of cytoplasmic inclusions shown in the diagram. Most neurones, however, contain filamentous mitochondria and 'blue' (wholly lipid) globules.

The cells of *Patella* studied by Lacy and Rogers (1956) and Lacy and Horne (1956) were evidently the large cells. They saw the pigmented or 'lipochrome' globules, and noticed that the globules in the centre of the cell are smaller than the others. They noticed that the long threads in the centre of the cell can be blackened by post-osmication in the Kolatchev technique, but did not remark that they can also be coloured by mitochondrial staining methods. The fact that certain mitochondria in a cell may differ from others in the capacity to reduce osmium tetroxide was shown recently by Meyer (1957) in his study of the neurones of *Hirudo*.

It is important to recognize that different authors have applied the term 'Golgi' to entirely different constituents of the gastropod neurone. Mousa (1950) and Boyle (1937) give the name of 'Golgi' to deposits of osmium silver on the surface of the yellow globules. I have shown that in *Helix* the curved rods or 'dictyosomes' seen in Weigl (Mann-Kopsch) preparations are artifacts caused by the modification of the 'blue' globules. Lacy and Rogers (1956) and Lacy and Horne (1956), on the contrary, regard the long threads of the central region of the neurone of *Patella* as the 'Golgi elements', presumably because they are easily osmicated. They brought forward no histochemical evidence, however, of any chemical similarity to any object in vertebrate neurones described by Golgi. Blackening by post-osmication gives no histochemical information, since a very wide variety of chemically unrelated substances are capable of reducing osmium tetroxide (Bahr, 1954).

It seems best to describe objectively the distribution, size, shape, and

structure of cytoplasmic inclusions, their reactions to vital dyes, and their composition as revealed by reliable histochemical tests. It would not appear that anything is gained by using the name of Golgi in describing these structures.

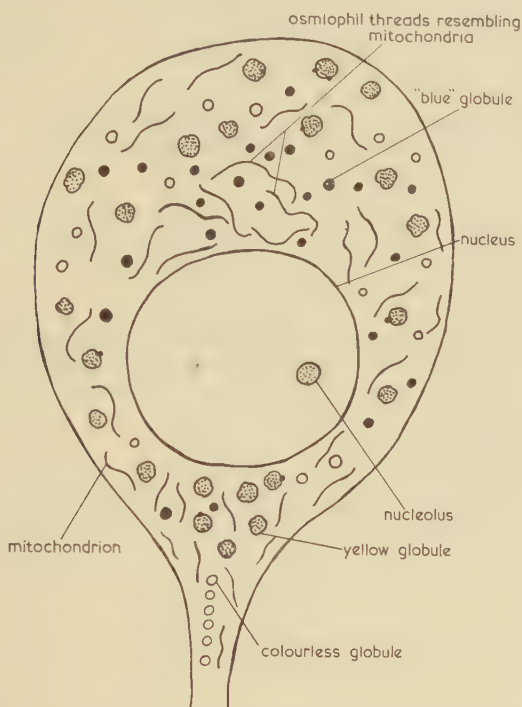


FIG. 3. Diagram showing all the kinds of cytoplasmic inclusions found in the neurones of *Helix aspersa*, *Limnaea stagnalis*, and *Patella vulgata*

Several authors have described secretory processes in these cells, but what they say is hypothetical. I have no concrete evidence of the time-sequence of the supposed stages.

I have great pleasure in acknowledging my debt to Dr. J. R. Baker for suggesting and supervising this investigation, and to Professor A. C. Hardy, F.R.S., for providing me with facilities for working in his Department.

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APPENDIX

Summary of cytochemical tests used and results obtained

<i>Test applied</i>		<i>Results obtained</i>	
<i>Name of test</i>	<i>Reference</i>	<i>Yellow globules</i>	<i>'Blue' globules</i>
Standard Sudan black	Baker, 1944, 1949, 1956b	+++	+++
Sudan IV	Herxheimer, 1910	+++	+++
Windaus's	Lison, 1953	o	o
Liebermann's	Lison, 1953	o	o
Fischler's	Pearse, 1954	o	o
Acid haematein (AH)	Baker, 1946	++	++
AH, pyridine extraction	Baker, 1946	o	o
Ciaccio's	Lison, 1953	+++	+++
PAAS	Pearse, 1954	++	+
PFAS	Pearse, 1954 Lillie, 1952	++	+
Casselman and Baker's for cerebroside	Casselman and Baker, 1955	+++	++
Casselman and Baker's for cerebroside control	Casselman and Baker, 1955	o	o
Nile blue	Cain, 1947	+ pale purple	o
Cain's plasmal reaction	Pearse, 1954	+ or uncertain	o
H ₂ SO ₄ for carotenoids	Cain, 1948	+++	o
PAS	Pearse, 1954	+++	o or +
PAS control	Pearse, 1954	o	o
PAS after saliva digestion	Pearse, 1954	++	o
Feulgen's	Feulgen and Rossenbeck, 1924	o	o
Feulgen's control	Feulgen and Rossenbeck, 1924	o	o
Bignardi's for neutral polysaccharide	Bignardi, 1940	o	o
Sakaguchi's	Baker, 1947	o	o
Hg/nitrite	Baker, 1956a	+	o
Coupled tetrazonium (C.T.)	Pearse, 1954	++	+ ? uncertain
C.T. after performic acid	Pearse, 1954	++	o
C.T. after dinitrofluorobenzene	Pearse, 1954	o	?
Basiphilia	—	+++	+++
Gomori's for alkaline phosphatase	Gomori, 1952	+	o
Metachromasy	+	o	o
Pyronin/methyl green	Jordan and Baker, 1955	orange	o

KEY: +++ = strong reaction; ++ = moderate reaction; + = weak reaction; o = negative.

A Histochemical Study of the Pigment Cells of the Leech, *Glossiphonia complanata*

By S. BRADBURY

(From the Cytological Laboratory, Department of Zoology, University Museum, Oxford)

With one plate (fig. 1)

SUMMARY

Glossiphonia complanata was found to contain two types of pigment cell. One is very large, usually globular in section, and is found in the deeper layers of connective tissue near the intestinal caeca. The second type is smaller and occurs only in the subcutaneous region of the body.

The large pigment cell contains very many, regular, pigmented spheres, each of which is about 3μ in diameter. These spheres have a proteinaceous substrate which is partly composed of tyrosine, arginine, and perhaps some histidine. Histochemical and other tests indicate that the yellowish pigment contained in the spheres is almost certainly a tetra-pyrrol compound, possibly related to the vertebrate bile pigments. The pigmented spheres may contain 'masked' iron. It is suggested that these pigmented spheres represent the accumulated waste products of haemoglobin breakdown, and so constitute a 'kidney of accumulation'.

The subcutaneous pigment cell is typically stellate. Both the cell-body and its numerous branching processes are filled with small brown pigment granules which never exceed 1μ in diameter. This pigment is characterized by extreme insolubility and chemical inertness, suggesting that it is a melanin. It is these cells which are primarily responsible for the colour of the animal.

MANY authors since Cuénot (1891) have described the large pigment cells which occur deep in the connective tissue of *Glossiphonia complanata* and other rhynchobdellid leeches. Detailed studies have been published by Graf (1899), Scriban (1910), Abeloos (1925), and Bobin (1950), but a histochemical study by modern methods does not seem to be available.

It is the purpose of this paper to give the results of such a study, both of the large deep-seated pigment cells, and of the smaller pigment cells which are found in the connective tissue immediately below the epidermis.

MATERIAL AND METHODS

Specimens of *G. complanata* were collected in the Oxford region and kept in the laboratory until required. Much of the work was done on sections of fixed material because of the difficulties in obtaining separate living cells (Bradbury, 1956b); details of the histochemical tests are not given in the paper but are set out in an appendix. As well as *in situ* histochemistry, chemical and spectroscopical studies were made of pigment solutions extracted from the leeches.

THE LARGE PIGMENT CELL

Morphology

These cells occur in the deeper layers of the connective tissue. Bobin (1950) has shown that they are found in the region between the base of the pharynx and the ventral sucker, with especial concentrations around the caeca of the stomach and intestine. The cells appear round or oval in section, often with a diameter of over 100μ . Bobin succeeded in isolating some of these cells and showed that they were globular or pear-shaped. During the present study it proved possible, by the use of gentle maceration, to separate the gut and much of the adjacent connective tissue and mount them on a slide. The large pigment cells were clearly visible and their shape and size agreed with the account given by Bobin.

In many of the sections studied, very little ground cytoplasm was seen in these cells, as the whole body of the cell appeared packed with very many pigmented spheres (fig. 1, A, B). The spheres are very regular; they mostly have a diameter of about 3μ . The pigment contained in or on these spheres is yellowish-brown or green when seen in unstained sections. The cells rarely show a nucleus, but when one is included in the plane of section it is seen to be oval, about 25μ by 12μ , with a very prominent nucleolus approximately 5μ in diameter. Occasionally a nucleus is found with a very irregular outline; it appears to be pushed up against one of the cell-walls by the pigmented spheres. It is very probable that these represent the 'secondary nuclei' noted and figured by Bobin in her recent paper. From the study of serial sections she concludes that the adipose cells may under some circumstances transform into these pigment cells by the gradual accumulation of the pigmented spheres in their cytoplasm. Bobin gives a schematic representation of such a cell cut in perfect longitudinal section, showing the transformation of the cytoplasmic structures typical of an adipose cell to those characteristic of the pigment cell. In the present work some sections cut longitudinally and coloured with Sudan black showed pigment cells with a very similar appearance to this ideal. At one end were large fat drops coloured intensely by the Sudan black; these graded into an intermediate zone with fat drops among pigmented spheres, whilst at the other end of the cell only pigmented spheres were seen. One such cell is shown in fig. 1, C. These observations may be interpreted as

FIG. 1 (plate). A, large pigment cell, seen in a section of the lateral coelomic sinus region of *Glossiphonia*. Note the regularity of the pigmented spheres.

B, a similar section to that shown in A, but stained with iron haematoxylin to show the slight basiphilia of the pigmented spheres.

C, an adipose cell which contains numerous pigmented spheres. The plane of the section includes typical adipose structures, an intermediate zone, and pigmented spheres.

D, a section through the superficial region of the body of *Glossiphonia*. Note that the stellate pigment cells form a complete layer just below the epidermis.

E, stellate pigment cells. This slide was prepared by the argentaffin technique; the pigment granules in these cells are strongly reducing, whilst those in a large pigment cell do not show this property.

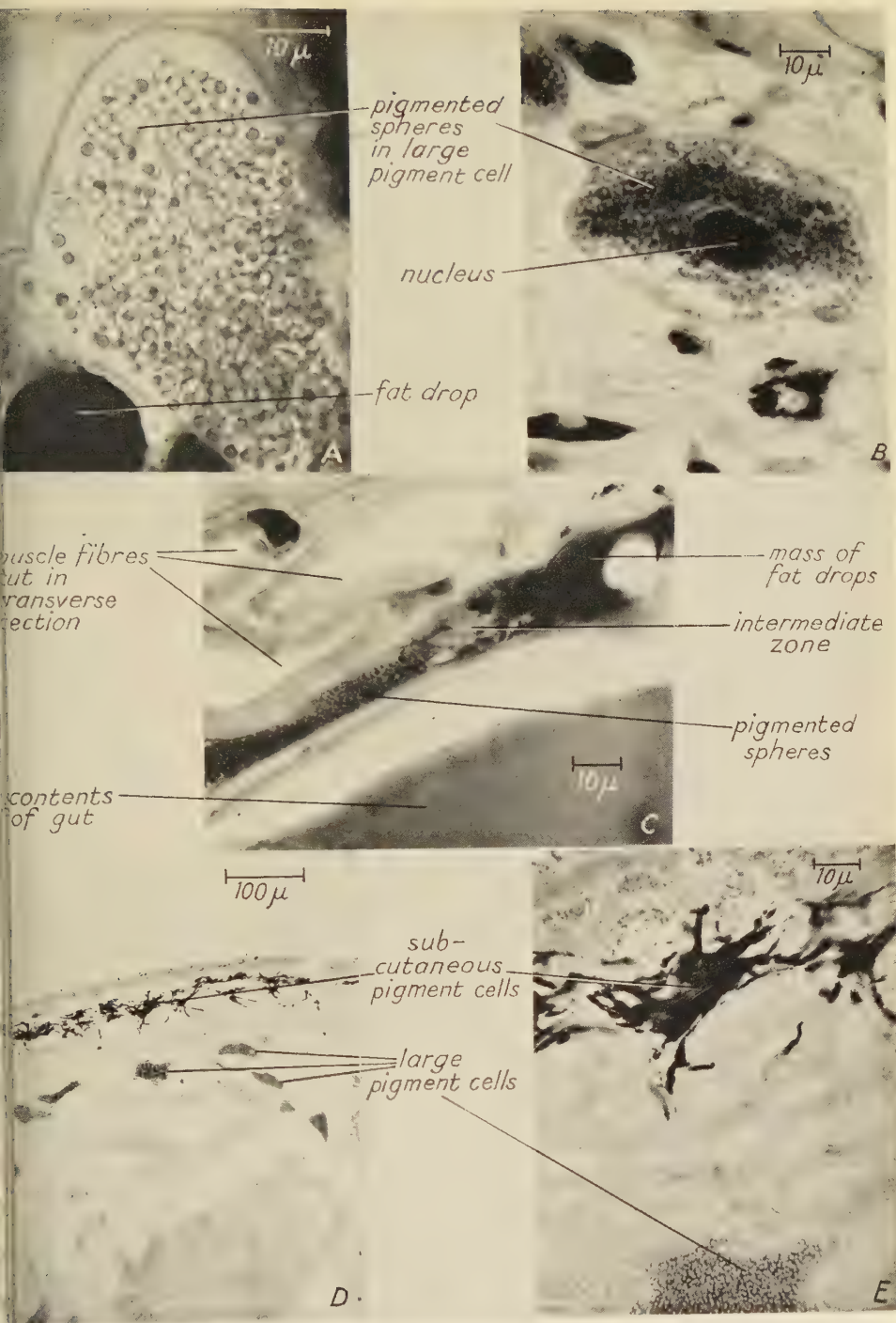


FIG. 1
S. BRADBURY

supporting Bobin's idea that there is no sharp distinction between the adipose cells on the one hand, and the pigment cells on the other.

The presence of 'residual lipid' as noted by the same author was confirmed. This lipid will colour with Sudan black and often appears as irregular masses. Mitochondrial techniques show the mitochondria to be rod-like, with a length of about 7μ and a diameter of 1μ . They tended to be scattered around the periphery of the cell, but were not very numerous.

Some cells were noted beneath the dorsal surface of the body which resembled the large pigment cells but were smaller and contained many extremely small pigment granules. The latter appeared rather yellowish in colour and were never larger than 1μ in diameter. Few observations were made on these cells, but they seem to correspond to the 'cellules blanches' noticed by Bobin (1950, pp. 73, 78). It would appear from the histochemical studies that this pigment differs significantly from that in the larger and more deeply situated pigment cells, but closely resembles that in the subcutaneous pigment cells.

Histochemistry

In most standard histological preparations, the pigmented spheres in this cell do not take up either acid or basic dyes, but appear in their natural yellowish colour. After fixation in Zenker's fluid, however, the spheres will colour feebly with dye lakes such as that of haematein, thus suggesting that they possess some basiphil components; this property was noticed by earlier workers. The basiphilia may be due to the pigment itself, or more probably is a property of some substrate to which the pigment is bound.

Lipids. Details of the tests for lipids which were used in this work are given in the appendix. It is seen that no positive result could be obtained with Sudan black or Sudan IV, either at room temperature or at 60°C . This makes it seem very unlikely that there is any free lipid in the pigmented spheres. Positive reactions were obtained in some instances from small, rather irregular masses situated among the pigmented spheres (the 'residual lipid' noted by Bobin, 1950). By the use of the acid haematein test (Baker, 1946) it can be shown that this residual lipid is a phospholipid.

A positive reaction to the Sudan black test was also given by the peripheral cytoplasm; this appearance exactly paralleled that of the cytoplasm of the adipose cell of the same animal. In the latter cell it was found that a positive reaction to Sudan black was given by the fat drop, the so-called 'surround', and also to a considerable extent by the ground cytoplasm (Bradbury, 1956b). This observation taken alone is not significant, but when considered in the light of all the other pieces of evidence which will be given in the paper, it seems to lend further support to Bobin's views on the origin of these pigment cells.

There appeared to be a possibility that the pigment represented some form of modified lipid, i.e. it was one of the pigments generally termed a 'lipochrome'. If this were the case, it seemed likely that free lipid would be detected

in the pigment spheres of some of these cells, or, alternatively that some lipid material might be revealed by unmasking techniques. In none of the cells studied was there any evidence for the existence of free lipid in the pigmented spheres. As recommended by Ciaccio (1926), unmasking was attempted both at the time of fixation by the use of a fixative such as Da Fano's fluid, or by the subsequent action of a 1% phenol solution for 24 h in the oven at 37° C. These treatments were followed by coloration with Sudan black in the normal way. It was not possible to show the presence of any lipid in the pigmented spheres, even after the unmasking with phenol; this makes it unlikely that the pigment may be considered to be a lipochrome.

A positive result was obtained with the performic acid / Schiff (PFAS) technique. This normally suggests the presence of unsaturated lipids, but since no lipid material was demonstrable in the pigmented spheres, it seems that this reaction must be the consequence of unsaturation in some other component of the pigmented spheres.

Carbohydrates. The tests for carbohydrates were negative with the pigmented spheres in this cell and it may be concluded that they contain no free carbohydrate. A positive PAS reaction was, however, obtained from the little residual cytoplasm situated round the edge of the cell. This reaction persisted after slides had been incubated in saliva at 37° C for one hour, so that it is not due to the presence of glycogen. Further, no metachromasy was seen when sections were stained in a solution of toluidine blue, but after sulphation with concentrated sulphuric acid for a very short time, as recommended by Lison (1953), the PAS-positive material became intensely metachromatic. This seems to suggest that the reaction is due to the presence of some neutral mucopolysaccharide.

Proteins and amino-acids. The large size of the pigmented spheres in this cell, together with their perfect regularity, suggest that there is some kind of supporting framework to which the pigment is bound. This view is supported by the fact that it is possible to bleach the pigment by a prolonged exposure either to strong hydrogen peroxide or to acids and alkalis, and if unstained sections are examined after such a treatment, the large pigment cells are seen to contain colourless spheres of a similar size to the pigmented ones which previously filled the cells. These colourless spheres may be supposed to represent the supporting framework of the pigment, and it is on such bleached preparations that all the histochemistry of amino-acids was done. When the Sakaguchi test as adapted to histochemical use by Baker (1947) was tried, a fairly strong positive result was obtained, the spheres colouring much more strongly than the ground cytoplasm. A similar result was given by the same author's Hg / nitrite test for phenols (Baker, 1956). It thus seems possible to conclude that the structural basis of the spheres is protein, and that it contains appreciable quantities of both arginine and tyrosine.

The 'coupled tetrazonium' reaction (Danielli, 1947; Pearse, 1954), together with the blocking reactions suggested by Pearse, was applied to this material. A strongly positive reaction was given by the spheres in this cell, with a

without pre-treatment with performic acid. After benzylation, the reaction was completely negative, but after treatment with dinitrofluorobenzene there was a positive reaction, though it was much reduced in intensity when compared with slides which had not been treated with this reagent. This reduction in intensity may perhaps indicate that a partial blockage was taking place. These results suggest that there are appreciable amounts of histidine and tyrosine present in the spheres, together with some arginine. It may thus be supposed that the spheres are primarily protein, to which the yellowish brown pigment is attached.

Nucleic acids. When the pyronin / methyl green technique (Jordan and Baker, 1955) was used there was no coloration of the pigmented spheres, which appeared in their natural colour, but the ground cytoplasm around the edge of the cell was strongly stained with the pyronin. By the use of the treated saliva technique (Bradbury, 1956a) this coloration could be completely prevented, so that this staining with pyronin may be considered to be due to the presence of RNA. The nuclei in the large pigment cells often appeared unusual, in that they coloured to a much greater extent than normal with pyronin; this could perhaps be due to their low content of DNA. The Feulgen reaction gave a strongly positive result for the nuclei which appeared among the pigmented spheres, but those which were found near the cell periphery coloured only weakly, and showed much vacuolation. It was these nuclei which were abnormal in their reaction to the pyronin; they may well be the degenerating nuclei noted by Bobin (1950), which have lost the greater part of their DNA.

Pigments; unclassified tests. The histochemistry of the pigments is still in a rather unsatisfactory condition. This is due largely to the lack of knowledge of the exact chemical composition of many of the pigments. As a result, it is not easy to obtain a definite diagnosis of the nature of a pigment by histochemical methods alone. Certain results can be obtained by using histochemical tests on the pigment in its original position and these, when taken in conjunction with studies of the extracted material, enable one to obtain some idea of its nature.

Unstained sections of the material were mounted and examined by ultraviolet microscopy. The pigmented spheres showed a strong brownish fluorescence which contrasted vividly with the bluish-white fluorescence of the background. This test excludes the possibility that the pigment of the spheres may be a porphyrin, as these compounds show a characteristic red fluorescence.

A further means of identifying pigments is by a study of their absorption spectra. This was attempted in the first instance by the use of the eyepiece spectroscope. It was found that the image of the large pigment cell could be made to occupy the whole of the slit of the instrument; this allowed its spectrum to be examined. The pigment did not show any absorption bands in the visible region of the spectrum, though there was much general absorption in the blue and violet region below about 500 m μ . These observations were checked by using solutions of the pigment and measuring the absorption at

different wavelengths with the spectrophotometer. These results will be considered in the next section.

The solubility of the pigment was tested on formalin-fixed material by immersing unstained sections in different solvents and examining the sections at regular intervals. It was found that water, ether, and chloroform had no effect at all, even after prolonged immersion; 70% alcohol and carbon disulphide each showed some slight extraction of the pigment after about 48 h, whilst glacial acetic acid or a 0.1 N solution of sodium hydroxide removed almost all the pigment in less than 24 h. In the slide which was acted upon by the alkali most of the pigment was extracted in about 1 h.

Hydrogen peroxide was effective in decolourizing the pigmented spheres but in this case the action might be due either to a solvent effect or to an oxidation of the pigment to some colourless compound. These solubilities could be explained by postulating that the pigment was in fact a bile pigment, a supposition put forward by Abeloos (1925) and Juga (1931). Abeloos found that the pigment in *Glossiphonia* had solubilities very similar to those listed above. Verne (1926) in his book on pigments remarks that bile pigments are soluble in chloroform and also, to a certain extent, in carbon disulphide. Biliverdin would also, according to this author, be soluble in alcohol. The other solubilities (water, acids, alkalis, ether) are in accord with those of a bile pigment. He states that bile pigments are characterized by the fact that they are 'bien soluble . . . dans l'acide acétique avec une teinte bleu-vert'. This is also true for alkalis and corresponds exactly to the results obtained in the experiments with whole animals to be considered on p. 307.

As a bile pigment was suspected, the two available confirmatory tests for bile pigments were used. The tests, which are said to be specific, are the Gmelin reaction (Tiedmann and Gmelin, 1826) and the Fouchet reaction, as quoted by Juga, 1931; Cole, 1955). In the case of Gmelin's test the chemical basis has been studied in some detail (With, 1954; Lemburg and Legge, 1949), and a positive result with this test may be taken as diagnostic for the presence of bile pigment. Both Abeloos (1925) and Juga (1931) obtained a positive Gmelin reaction from the pigmented spheres studied in sections; this was tried many times in the course of the present study, but in no case did the reaction work on sections. Fouchet's test, on the other hand, when tried on gently macerated material, gave a very marked blue-green colour which was localized in the pigment cells. This colour may be considered as a strongly positive reaction.

Pearse (1954) mentions a further reaction for bile pigments. This is Stein's test, based on the oxidation of the pigment to green biliverdin by means of a dilute iodine solution. This reaction is capricious, and no definite results were given by the pigment cells.

There was a possibility that the pigment might be a melanin, though in view of its solubilities this seemed rather unlikely. The melanins are characterized by their general inertness, but one of the characters which serves to differentiate them from other pigments is their capacity for reducing silver in

alkaline solution. This test was carried out according to the directions given by Pearse (1954), with the result that the large pigmented spheres were found to be non-reducing. The small pigmented spheres in the 'cellules blanches' already mentioned were found to have fairly strong powers of reducing alkaline silver solutions, which seems to indicate that this particular pigment is very different from that in the large pigment cells.

Abeloos and Juga both stated definitely that there was no iron in these pigmented spheres. This was checked during the present work by the use of both Perls's technique and by microincineration. In a previous paper (Bradbury, 1955) it was stated that the pigmented spheres gave a weak Perls's reaction; this was again noticed in several animals, though one or two gave a much more marked reaction than the rest. If the test were preceded by a treatment with very strong (100 vol) hydrogen peroxide, this positive reaction was much increased, suggesting that perhaps the iron is normally present in a masked form. Microincineration gave a positive result in all the sections examined, and as this test is independent of the state of the iron, it seems possible to say that iron is generally present in the pigmented spheres, though usually in a form which is not easily demonstrable with the standard chemical reactions. This conclusion is opposed to that reached by Abeloos and Juga, though as far as can be ascertained, neither of these workers used the microincineration technique.

Extracted pigment. When it was found that glacial acetic acid and caustic soda would remove the pigment from this cell, it was decided to attempt extraction on a larger scale in order to obtain sufficient of the pigment for spectrophotometric study. At the same time it was thought worth while to check the solubility in various solvents when large pieces of fresh tissue were used.

When fresh material was boiled with the solvents in a simple reflux apparatus, it was found that neither water, 70% alcohol, chloroform, nor a chloroform / methyl alcohol mixture extracted any pigment even after refluxing for 24 h. When the tissue was subsequently embedded and sectioned, it was found that the pigment was still present both in the large deep-lying cells containing the pigmented spheres and in the subcutaneous pigment cells. With carbon disulphide a yellowish solution resulted after refluxing for 24 h, but it was doubtful whether this represented a pigment from the pigmented spheres, because when sections of this extracted material were examined, these cells appeared to show no change in colour. When glacial acetic acid or 0.1 N sodium hydroxide was used as the solvent, the pigment passed into solution in less than an hour. These solutions were filtered to clarify them and then studied in the spectrophotometer. The absorption in the visible region of the spectrum is shown in fig. 2. A further sample was examined with a Unicam ultra-violet spectrophotometer so that the absorption in the region 420–500 m μ could be studied (fig. 3). Similar curves for a solution of mammalian bile are drawn on the same figures for comparison; the similarity of the two is obvious. With (1954) in his monograph on the bile pigments points out that

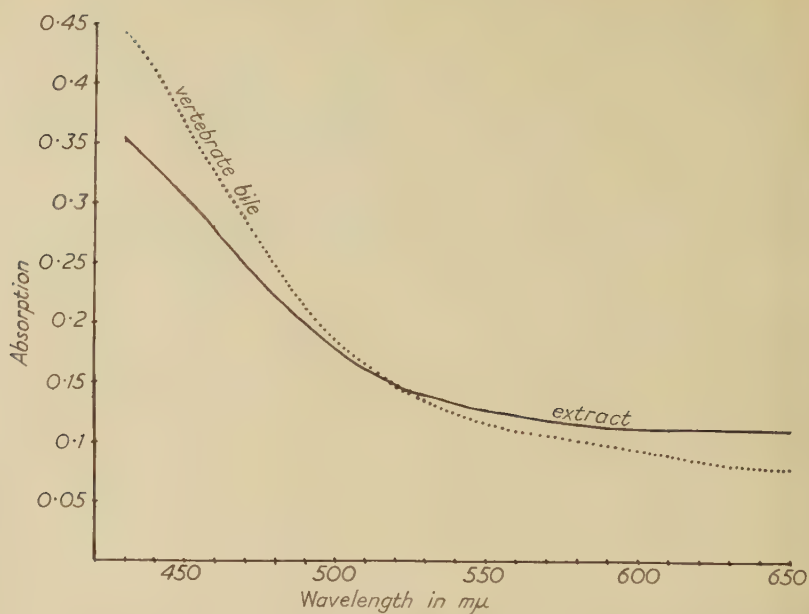


FIG. 2. Absorption spectra of an extract from *Glossiphonia* and of a solution of vertebrate bile pigment.

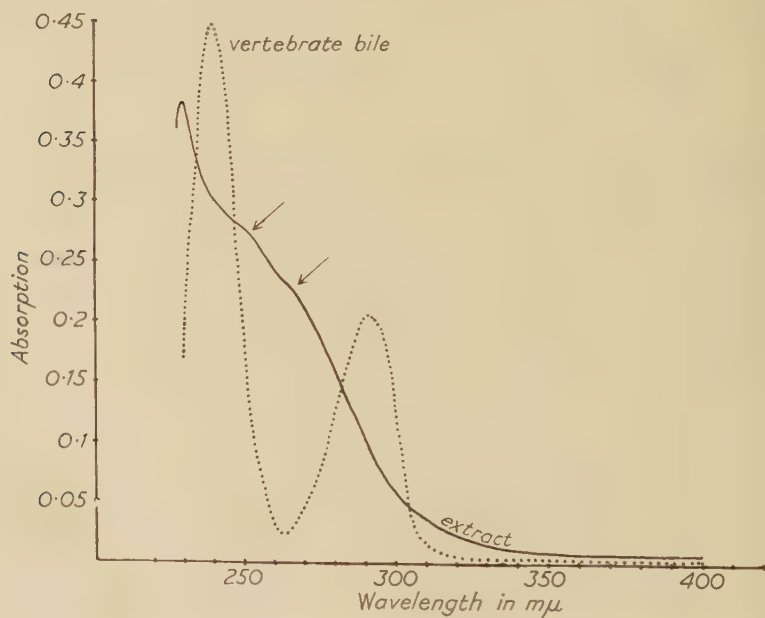


FIG. 3. Ultra-violet absorption spectra of an extract from *Glossiphonia* and of a solution of vertebrate bile pigment. Note the 'shoulders' on the curve at 255 $m\mu$ and 270 $m\mu$.

There are no pronounced absorption bands in the visible spectrum, but that bilirubin in alkaline solution shows a maximum absorption at $420\text{ m}\mu$, whilst urochrome has its maximum between 300 and $370\text{ m}\mu$. It is seen in the figure that both curves show a maximum around $240\text{ m}\mu$, but the strong maximum of vertebrate bile at $290\text{ m}\mu$ is not obvious in the extract. There are, however, suggestions of irregularities in the absorption curve of the extract at $255\text{ m}\mu$ and $270\text{ m}\mu$. These may well be ill-defined absorption peaks which are not more obvious because of the very dilute nature of the extract. They may, however, be due to the presence of some impurity in the pigment solution.

The Gmelin and Fouchet tests were performed on this aqueous alkaline extract. Positive results were obtained in both cases. As a result of the tests stated above, it seems possible to arrive at a fairly certain identification of the pigment in the large pigment cells. It does not belong to the group of melanins, it is fairly soluble in several different liquids and it does not have the power to reduce alkaline solutions of silver. Similarly, no trace of lipids could be found in the pigmented spheres, even after the most vigorous unmasking techniques, so that it seems unlikely that the pigment belongs to the lipochrome or lipid-derived pigments.

The solubilities and absorption spectra suggest that Abelson and Janda were correct in thinking that this pigment is one of the tetra-pyrrole derivatives, probably resembling the vertebrate bile pigments. In addition it must be noted that the ultra-violet fluorescence of this pigment is similar to that of vertebrate bile pigment; furthermore there was an association with a proteinaceous substrate which, as With (1954) points out, is always true of bile pigments. There was also a positive Gmelin and Fouchet's reaction from extracted samples of the pigment. On the other hand, the pigment was not soluble in chloroform or alcohol, which seems to show that it is not identical in every respect with a bile pigment. Stein's reaction and Gmelin's reaction when applied to sections did not give a positive result. As Pearse (1954) emphasizes, a negative result with these tests does not signify anything, as they are known to be capricious. Extracts did give a positive Gmelin's reaction, and as the chemistry of this reaction is known with some certainty (Remburg and Legge, 1949; With, 1954), it may be assumed that a positive result is diagnostic of a bile pigment.

It thus seems possible, after considering all the evidence, to draw the following conclusions. The pigmented spheres in the large pigment cells have a proteinaceous substrate; this contains arginine, tyrosine, and possibly some histidine. The pigment in these spheres is almost certainly a tetra-pyrrole compound, resembling, though not identical with, the bile pigment of vertebrates. It has been possible to show that the pigmented spheres may in some cases contain iron present in a 'masked' condition.

THE SUBCUTANEOUS PIGMENT CELL

Morphology. These cells are found in the connective tissue immediately below the epidermis. They are much more numerous beneath the dorsal

surface, but isolated groups of these cells occur below the epidermis of the ventral side. The cells are arranged to form a single layer and are so close together that their processes interlace (fig. 1, D, E). It seems that these cells are responsible for the general body coloration.

The actual cells are irregular in shape; they could perhaps be described as stellate. The body of the cell is about 20μ in diameter and both it and the numerous branching processes are entirely filled with pigment granules. It is noticeable that the finest processes of these cells may have pigment granules located in swellings along their length and often the process terminates in one

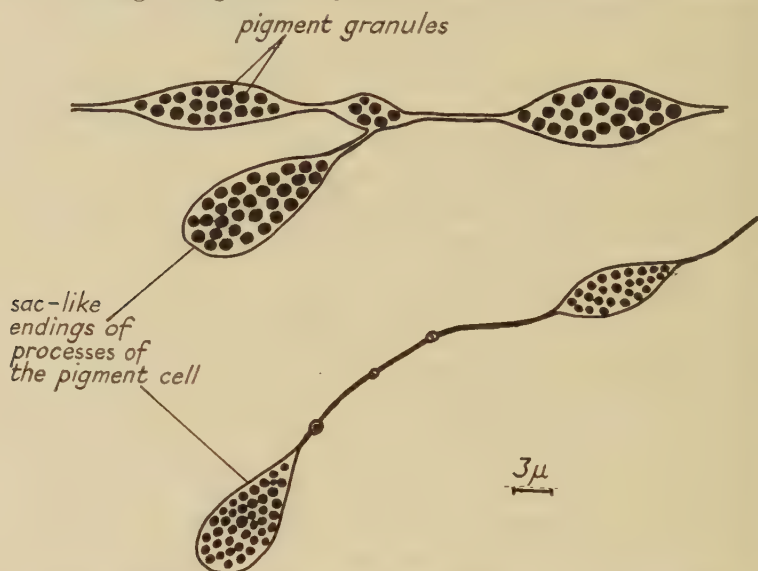


FIG. 4. Diagram to show the processes of the stellate pigment cell. Note the pigment granules contained in swellings and the sac-like ending of the processes.

of these swellings (fig. 4). The cells appear to contain only one nucleus which is oval and about 12μ long. The pigment granules are extremely numerous and are dark brown or yellowish brown; they are very much smaller than the pigmented spheres in the large pigment cell, for in no instance were they found to exceed 1μ in diameter, and usually the majority were less than 0.5μ across.

Pigment. It was found that the pigment granules in these cells were insoluble in water, 0.1 N NaOH , glacial acetic acid, 70% alcohol, ether, carbon disulphide, and chloroform; with hydrogen peroxide the pigment was bleached in about 48 h.

The pigment granules in this cell reduced alkaline silver solutions very strongly; this is one of the characteristics of melanins and was used for their identification by Bizzozero (1908). The granules also possess the power of reducing ferricyanide to ferrocyanide and so forming Prussian blue in the presence of ferric salts. This reaction is the Schmorl reaction and is characteristic of lipochromes, argentaffin cell granules, and melanins (Pearse, 1954).

All other tests applied were found to give negative results with the pigment granules of this cell (see appendix). It thus seems that this brownish pigment is characterized by an extreme insolubility and chemical inertness. Its properties seem to place it in the class of melanins.

DISCUSSION

Several suggestions have been put forward during the last 50 years to account for the origin and function of these large pigment cells in *Glossiphonia* and in other rhynchobdellid leeches. Cuénot (1891) considered that they were excretory, and acted as a 'kidney of accumulation'; Graf (1899), on the other hand, termed them *Stapelzellen* or reserve cells. He considered that they formed one of the functional stages in the progressive development of the 'excretophores' from the acidic cells which line the coelomic sinuses. Juga (1931) supported this idea of their derivation, including them among her category of 'chromatocytes', but she seems to think that these cells are specifically excretory. Both of these authors considered that the dermal pigment cells are derived from the large deeper-lying pigment cells.

Scriban (1910) and Abeloos (1925) both reject Graf's idea of the origin of the large pigment cells, because they did not find that the 'acid' cells possessed any amoeboid powers, as suggested by Graf and Juga; instead, the large pigment cells were considered to arise by independent differentiation from an embryonic connective tissue cell. Abeloos also came to the conclusion that these cells acted as a kidney of accumulation.

Bobin (1950) puts forward a very strong case for supposing that the pigment cells have their origin from some of the large adipose cells which form such a prominent feature of the connective tissue of *Glossiphonia*, but she does not seem to put forward any hypothesis as to their function. From results obtained during the course of the present study, it seems that Bobin is correct in supposing the pigment cells to arise from adipose cells, as stages resembling adipose figures in her paper have been seen in my preparations.

The pigment in these cells seems almost certainly to be a tetra-pyrrol compound closely related to the vertebrate bile pigments. This would accord with the views of Cuénot and Abeloos that the pigmented spheres are the products of haemoglobin metabolism. It must be supposed that the protein of the ingested blood would be freed from the haem prosthetic group, which would then be further degraded to give a porphyrin and free iron. The porphyrin in turn would be broken down to a linear tetra-pyrrol compound which would accumulate as the pigment in the coloured spheres. Wigglesworth (1943) found that in the blood-sucking bug *Rhodnius* some of the ingested blood is denatured to give biliverdin which is subsequently either excreted through the gut, or stored in the pericardial cells which presumably act as a kidney of accumulation.

It has been shown (Bradbury, 1955) that free iron can be detected in great quantities in the connective tissue of *Glossiphonia* after a meal of blood containing haemoglobin. Most of this iron is localized in the adipose cells, but some does

appear in other regions of the body. It might reasonably be supposed that the actual breakdown and metabolism of the haemoglobin is taking place in the adipose cell. Bobin's conclusion that the pigmented spheres have their origin in this cell accords very well with the present hypothesis. These bodies would be regarded as the end products of the digestion of haemoglobin which are of no further use to the animal and accumulate in the cytoplasm of this cell. Eventually, when they are present in great quantity in any one cell, the cytoplasm degenerates, leaving an 'envelope' enclosing a mass of pigmented spheres. It seems that the view proposed so long ago by Cuénot is perhaps correct and the pigment cells are acting as a kidney of accumulation.

The failure to detect porphyrin in any of these cells could be explained by assuming that this stage in the breakdown of haemoglobin was very rapid, so that these compounds would only be present in the cells for a very short time. The presence of some 'masked' iron in the coloured spheres might be expected if they represent the metabolic waste-products of a process involving iron-containing compounds; it might be that the process of breakdown was not complete in a particular animal at the time of killing. This could also explain why Juga and Abeloos were unable to detect any iron in these cells.

In the paper already quoted (Bradbury, 1955) it was pointed out that the 'acidic' cells of the coelomic epithelium contain very little iron, even after the leech has been fed; this is in contrast to the adipose cells, which contain very large amounts of iron. This observation seems to furnish further indirect evidence against the views of Graf and Juga that the coelomic epithelium gives rise to the pigment cells. If the latter are concerned in the excretion or accumulation of unwanted iron-containing pigment and originate from the coelomic epithelium, then it might reasonably be supposed that the coelomic cells would show incipient pigment formation or accumulations of iron; these have not been observed in the present work. On the other hand, if Bobin's idea is correct and the pigment cells are closely related to the adipose cells, then there is a very good correlation between the presence of iron and the pigment, both of which are possible waste-products of the metabolism of haemoglobin and similar compounds.

It will be difficult to obtain definite proof of this, but it is hoped that further experimental work which is planned will help to provide a better understanding of the functions of both the adipose cell and the pigment cell.

One further point which is apparent from the present work is that there does not seem to be any direct relationship between the large, deep-lying pigment cells and the subcutaneous pigment cells. They differ both in structure, and so far as can be ascertained, in chemical composition of the pigment granules.

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APPENDIX

Test	Reference	Large pigment cell	Subcutaneous pigment cell
Colour	—	Yellow, yellow brown	Dark brown
Basiphilia	—	+	—
U.V. fluorescence	—	Brownish	—
Absorption spectra	—	No bands in visible range. Blue and violet absorbed	—
Sudan black	Baker, 1944, 1949	o + ground cytoplasm ++ residual lipid	o
Sudan IV	Herxheimer, 1901	o	o
Sudan black at 60° C	—	o + ground cytoplasm ++ residual lipid	o
Sudan black after Da Fano's fluid	Ciaccio, 1926	o	o
Sudan black after phenol	—	o	o
Acid haematein	Baker, 1946	o ++ residual lipid	o
Acid haematein, pyridine extraction	—	o	o
Performic acid Schiff	Pearse, 1954	+++	o
Plasmal	Cain, 1949a, 1949b	o	o
Windaus	Lison, 1953	o	o
Liebermann	—	o	o
Pyronin methyl-green	Jordan and Baker, 1955	o nuclei ++ MG some ++ pyr. ground cytoplasm + pyr.	o
Pyronin methyl-green + saliva	Bradbury, 1956a	o	o
Feulgen	Feulgen and Rossenbeck, 1924	+ nuclei	+ nuclei
Feulgen control	—	o	o
PAS	Pearse, 1954	o ++ ground cytoplasm	o
PAS + saliva	—	o	o
Toluidine blue	Baker, unpublished	o	o
Toluidine blue + sulphation	Lison, 1953	o ++ ground cytoplasm	o
Sakaguchi	Baker, 1947	++	o
Hg nitrite	Baker, 1956	++	o
Coupled tetrazonium	Danielli, 1947 Pearse, 1954	+++	o
CT + benzylation	—	o	—
CT + performic acid	—	+++	—
CT + dinitrofluorobenzene	—	+	—
Alkaline silver	—	+	+++
Schmorl	—	+	+++
Perl's	Gomori, 1952	+++	o
Incineration	—	+++	o
Gmelin	Tiedmann and Gmelin, 1826	o in sections +++ with extracts	o
Fouchet	Quoted in Cole, 1955	+++	o
Stein	Pearse, 1954	o	o
Hydrogen peroxide bleach	—	24-48 h	c. 48 h

Key to the appendix: o = negative reaction; — = no observation; + = weak reaction; ++ = moderate reaction; +++ = strong reaction.

Observations on the Structure of Hydra as seen with the Electron and Light Microscopes

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With 6 plates (figs. 1 to 6)

SUMMARY

Sections of hydra studied with the electron microscope show various structures which have been identified by referring to control histological sections and to previous descriptions. Certain features have also been examined in frozen-dried sections under the light microscope.

In the ectoderm, epithelio-muscular cells contain various organelles, and also smooth longitudinal muscle-fibres with which mitochondria may be associated. The so-called 'supporting fibres' appear to be thin bundles of muscle-fibres. Although points of contact exist between muscle-fibres, there appears to be no cytoplasmic continuity. The muscle-fibres insert on the mesogloea, and appear to be separated from it by two membranes, one belonging to the cytoplasm surrounding the muscle-fibre and the other to the mesogloea.

The mesogloea is extracellular and quite distinct from the intracellular muscle-fibres. It appears granular and sometimes presents an indistinct fibrous background. In frozen-dried material the mesogloea stains blue with Mallory's method, while the muscle-fibres stain red.

Two main types of cells are found in the endoderm. Among these, some of the digestive cells contain transverse muscle-fibres, but they are less distinct than the longitudinal ectodermal fibres. Otherwise the digestive cells vary much in structure, but generally they contain vacuoles and their free surface is thrown into villi covered with small granules. The 'foamy gland cells' are filled with much larger vacuoles containing granular material. The vacuoles are discharged together with portions of cytoplasm, and at this stage lamellated double membranes and mitochondria appear between the vacuoles. Both types of cell possess two flagella, which show a typical ultrastructure and are surrounded by a thick membrane.

Various other cells of the ectoderm are distinguished by their characteristic appearance. Cnidoblasts, for instance, have been found to contain an extensive system of intercommunicating vacuoles bounded by membranes, and do not resemble the interstitial cells. In unexploded penetrant nematocysts the tube is preformed and the butt and stylets can also be seen. The special gland-cells of the pedal disk show large, electron-dense granules which are extruded from the cell without any cytoplasm. A relatively thick homogeneous layer on the surface of the pedal disk is distinguished by the electron microscope.

INTRODUCTION

ALTHOUGH the two-layered anatomy of hydra is well known, the structural units of which it is composed are so small that use of the light microscope has never fully elucidated their arrangement. The far greater resolution afforded by the electron microscope enables some new observations

to be made on the fine structure of this animal, and provides further information about the nature and distribution of the mesogloea, of the muscle-fibres and of various kinds of cells composing the ectoderm and endoderm.

MATERIAL AND METHODS

Electron microscopy

A large, colourless variety of *Pelmatohydra oligactis* was employed in the electron microscope study. Animals were fixed for 10 min to 1 h in Dalton's fixative (Dalton and Felix, 1955), a solution which contains 1% OsO_4 , 1% $\text{K}_2\text{Cr}_2\text{O}_7$ at pH 7.2, and 0.85% NaCl . Pieces of hydra were also fixed in buffered 1% OsO_4 in Ringer's solution for similar lengths of time. The best results were obtained after short fixation in Dalton's fluid, and most of the electron micrographs are taken from material treated in this way.

Certain animals were placed directly in the fixative and contracted immediately. These were preserved in a contracted state. Others, which were first anaesthetized with $\frac{1}{2}$ % chloral hydrate, remained relaxed and were neither greatly contracted nor extended when fixed. Some of the anaesthetized hydras were divided into parts (hypostome, body, pedal disk) before fixing.

After fixation, the specimens were washed in distilled water, placed in 70% alcohol, dehydrated, and then embedded in a partially polymerized mixture of one part of methyl methacrylate to three of butyl methacrylate. This was polymerized at 45° C with benzoyl peroxide as the catalyst. Ultra-thin sections (about 300 Å thick) were cut with a modified Minot rotary microtome equipped with a glass knife, or with the Servall Porter-Blum microtome. Sections were taken in transverse, longitudinal, sagittal, and oblique planes. Most of the observations were made on transverse and longitudinal sections of the body, these being the specimens whose orientation and appearance were simplest to interpret. The sections were mounted on copper mesh grids, which were inserted into a Philips EM100 or an RCA-EMU type electron microscope, without removing the plastic. Negatives were exposed at a magnification of 1,000 to 6,000 times and enlarged photographically to the desired size. The final magnifications are approximate.

Light microscopy

In order to assist the recognition of structures seen with the electron microscope, control sections were examined regularly under phase contrast. Ordinary histological preparations of white and brown *P. oligactis* were also of help in orientating the electron micrographs.

Frozen-dried sections of a brown variety of *P. oligactis* have been studied with the light microscope. After freezing-drying (see Bell, 1956), specimens were placed in methyl benzoate with 1% cellodine; the normal procedure for double embedding was followed (Pantin, 1948). Section ribbons were flattened over Baker's formaldehyde-calcium (1944), on which they were left floating overnight at room temperature for post-fixation of the tissues. After two or three changes of distilled water, the ribbons were floated on to albumenized

slides, left to dry overnight, and stained by Mallory's trichrome method (Pantin, 1948). Although post-fixation may cause sections to shrink slightly, they become easier to handle and stain more clearly. It may be noted that freezing-drying, like osmium fixation, does not produce specimens uniformly free from artifact, and that certain regions are much better preserved than others.

RESULTS

It should be borne in mind that all the observations recorded here have been made on fixed material. While the high magnification of the electron microscope allows fine structure to be described far more accurately than is possible with the light microscope, the relation of many structures occurring in fixed material to those found in living hydra has not yet been determined.

The ectoderm

Epithelio-muscular cells. Certain cells of the ectoderm possess large oval nuclei of an even granular texture (fig. 1, A, *nuc*). Their cytoplasm contains a large variety of organelles and inclusions, among which may be found small vacuoles bounded by membranes with apposed granules, structures showing folds extending a short distance into the interior of the organelle and which may be identified as mitochondria of moderate size (fig. 1, A, *mit*), and complex bodies consisting of adjacent smooth membranes, vesicles, and vacuoles. There are several other inclusions, usually seen as electron-dense bodies, such as, for example, a frequently occurring ellipsoidal structure which is distinguished from mitochondria by being smaller and much denser, and without internal folds (fig. 5, F, *org*). Another body, although only moderately dense, is also darker than the surrounding cytoplasm (fig. 1, A, *org*), and is round in outline with a folded or crumpled membrane. Large and small granules, within which further structure may be detected, are seen at the centre. This type of inclusion or organelle, while varying slightly, often occurs in endodermal digestive cells as well (p. 322). A granular material adhering to the outer surface of the ectodermal cells forms a thin coat over the hydra (figs. 1, A; 5, F, *surf*).

Within some of the cells may be seen closely packed bundles of fine fibrils arranged in parallel and running in the general direction of the column axis (fig. 1, A, *mf*). The fibrils are usually accumulated at the base of the cell (compare fig. 2, C, *ect*), but as in fig. 1, A, some may extend upwards above the nucleus. The appearance and disposition of the bundles of fibrils indicate that they correspond to the myofibrils of histologists (see von Gelei, 1924), and the cells in which they are found are therefore epithelio-muscular cells. A detailed consideration of the structure and orientation of these intracellular fibrils further confirms their identity (see p. 322).

Several authors have reported the presence also of supporting or skeletal fibres in the epithelio-muscular cells of hydra, especially in the region of the foot and gonads. They are believed to provide stiffness and elasticity, although

Mueller (1950) has suggested that some of them are in fact muscular. Now the 'supporting fibres' figured by von Gelei (1924) pass from the cell-body into the basal extensions of the epithelio-muscular cell and run alongside the muscle-fibres (compare fig. 6, B). They would thus correspond closely in arrangement to the fibrils which extend into the ectodermal cells as just described. We find that the fine structure of these fibrils is identical with that of the myofibrils at the base of the epithelio-muscular cells. 'Supporting fibres' have not usually been described as being present in the endodermal cells, and correspondingly we find that myofibrils in the endoderm are restricted to the base of the cells. Our observations, therefore, tend to confirm Mueller's suggestion that many 'supporting fibres' form part of the muscular system.

Mueller (1950) agrees with von Gelei (1924) and other authors, however, in believing that a distinct class of intracellular supporting fibres does exist, but our observations so far provide no evidence for this. It must be remembered that other investigators have used different methods from those in the present study, and that under the light microscope fine fibrillar structures are near the limit of resolution and difficult to interpret with certainty.

Cnidoblasts and developing nematocysts. Another kind of ectodermal cell occurs in groups, which are sometimes found next to the mesogloea. Each cell contains a large round body which is limited by a relatively thick homogeneous border, and shows dark particles scattered throughout the interior (fig. 4, A, *nem*). In other cases, these organelles may be even larger, and sections reveal that they are undoubtedly developing nematocysts. The structures in fig. 4, A, are therefore immature nematocysts at various stages of development, and the cells containing them are cnidoblasts.

The nucleus of each cell may be displaced by the developing nematocyst. Within the nucleus a very dense organelle showing small clear patches represents the nucleolus. It apparently does not have a thread-like or vesicular structure and is usually found towards the centre of the nucleus (fig. 4, A, *n, nuc*). Other electron-dense particles are also seen, scattered irregularly within the nuclear membrane.

In these cells numerous vacuoles of a moderate size are dispersed throughout the cytoplasm and intercommunicate with each other, often giving the appearance of channels. The vacuoles generally appear to be empty and are bounded by membranes. At high magnification, numerous fine granules are seen in and around the membranes, and the cytoplasm between two membranes may also appear granular. In some sections the cytoplasm immediately surrounding a nematocyst rudiment is particularly dense and shows a lamellated structure (fig. 4, A, *lam*). Bodies are also present whose internal folds and structure resemble those of the mitochondria seen in other cells, and they may therefore be identified as such (fig. 4, A, *mit*).

Although only one nematocyst occurs in each cnidoblast, sections of a group of developing cells show that the cytoplasm of adjacent cnidoblasts is apparently continuous at certain points along their borders (fig. 4, A, *con*). If this

appearance is not due to fixation artifact, it may indicate that cells in a group of cnidoblasts are in syncytial relation to each other. Such a syncytial arrangement of cnidoblasts containing immature nematocysts might ensure that the batteries of nematocysts in the tentacles developed in a co-ordinated manner. The orderly pattern of mature nematocyst types has been studied by von Gelei (1927) and Semal van Gansen (1951).

Nematocysts. An electron microscope study of hydra nematocysts has been made by Semal (1954a), who used whole mounts of discharged capsules. Four kinds of nematocysts are found. The parts characteristic of the stenotele, or penetrant type, are the capsule, leading into the shaft or basal portion of the butt and carrying projecting stylets, the distal part of the butt or conical piece, which may sometimes carry spines, and the elongated filament or tube, which extends from the conical piece and may either carry rows of spines (Hyman, 1940) or be devoid of armature (Semal, 1954a).

In sections through the capsule of a mature undischarged stenotele within a cnidoblast (fig. 3, B), the coiled and elongated filament (*t*) is found embedded in an electron-dense homogeneous matrix, which is presumably fluid coagulated within the nematocyst capsule. The filament is a narrow tube with homogeneous walls and a central lumen: it appears to be empty and to lack spines. The conical part of the butt is seen in figs. 3, B (*b*) and 3, D as two serrated structures on whose inner sides numerous dark overlapping spines are attached. Above these in fig. 3, B are the large stylets (*s*), within the basal part of the butt. The attachment of the butt to the capsule wall cannot be seen in these sections, but in fig. 3, D the distal end of the butt extends as the beginning of the nematocyst thread, which has been cut through.

Sections through capsules of another type of nematocyst also show structures composed of overlapping layers of electron-dense material (fig. 3, C, *sp*). They apparently represent sections through the barbed thread of a holotrichous isorhiza or large glutinant nematocyst. These barbs appear similar in structure to the spines on the butt of stenoteles.

The appearance of the tube in sections of undischarged nematocyst capsules shows that the filament is already present before the nematocyst discharges and that it is not formed by extrusion of fluid or magma as suggested by Lepner and his colleagues (1951).

Interstitial cells. Groups of small, rounded cells also occur in the ectoderm (fig. 1, C). They are numerous in some areas and absent from others, and appear to be the interstitial cells.

The nucleoplasm of these cells is very light, but it contains granules and an extensive network of dark substance, which may represent nucleolar material. In some nuclei this dense network may surround islands of light nucleoplasm. The cytoplasm is not very dense, and contains granules, mitochondria with internal folds, and round vacuoles of varying sizes (fig. 1, C, *vac*) to which granules may be apposed. In some sections a complex of smooth membranes and vacuoles is seen.

If the interstitial cells give rise to cnidoblasts (fig. 4, A; see Hyman, 1940),

they must undergo remarkable changes in organization, especially in the case of the nucleolus and of the cytoplasmic vacuoles.

Gland-cells. Groups of secretory cells also occur in the ectoderm of the foot region. Large vacuoles, each of which usually contains a dense ellipsoidal granule, are found towards the periphery of each cell (fig. 5, A, *vac, g*). There is considerable space between a granule and its vacuole, probably due to shrinkage during preparation of the tissue. The cytoplasm between vacuoles contains mitochondria. The outermost cell membrane is thick and finely granular, and is covered by an external layer of dark amorphous material (fig. 5, A, *al*).

These cells are apparently ectodermal gland cells of the pedal disk, which are the only kind found in that region and are characteristically filled with coarse granules (Hyman, 1940). Their appearance in frozen-dried material is seen in fig. 6, E, which shows part of a vertical section of the foot (compare fig. 5, A). The peripheral granules are seen again in tangential section in fig. 6, F. The amorphous substance seen in the electron micrographs is probably the adhesive material which cements hydra to the substratum and which is secreted by these gland-cells.

In some electron microscope sections the secretory granules are found outside the foot of the hydra as if they had just been extruded (fig. 5, A): they may rest in a depression in the outer cell-membrane or in the amorphous layer and give the impression that they have just passed out of the cells and are still clinging to the surface. The extrusion of granules may nevertheless be an artifact, as it is possible that they were forced out of the cell mechanically during preparation of the tissue.

The mesogloea

The mesogloea, which lies between the ectoderm and endoderm (figs. 2, A, B, C; 3, A; 5, E, *mes*), is non-cellular and usually appears light, although in some sections it is fairly dark. It is usually granular, and the granules may be close together or fairly scattered. In some preparations they seem to be resting on a background of extremely fine longitudinally orientated threads, and it is possible that some of the granules in the mesogloea represent cut fibrils. Other oblique sections reveal very fine threads which appear to be present throughout the mesogloea and give it a fibrillar texture (fig. 5, E, *mes*).

In frozen-dried preparations also the mesogloea sometimes appears to be fibrous in nature, although when poorly fixed it appears reticulate. If true fibres are present, this is a further likeness between the mesogloea of hydra and that of other coelenterates (see Chapman, 1953). The varying appearance of the mesogloea probably depends on the state of contraction in which the hydra is fixed.

The mesogloea appears to be bounded by two basement membranes, which separate it from the ectoderm and endoderm respectively. These membranes are not always easy to discern but may be seen in fig. 2, C (*doub*). They will be discussed further in considering the relation of the muscle-fibres and meso-

gloea (p. 324). The mesogloea also shows holes filled with a very light substance (figs. 2, A, C). As will be explained below, these represent oblique sections of cytoplasmic roots, which are extensions of the cytoplasm surrounding the muscle-fibres and penetrate the mesogloea (Semal, 1952).

The endoderm

The endodermal cells of hydra vary in structure and appearance according to the region of the animal as well as to the time since the last meal. Two main types of cell are concerned with the digestion and absorption of food: the gland cell, which pours secretion into the lumen to break down the food and makes its products available for intracellular and perhaps extracellular digestion, and the digestive cell, which absorbs the food products. Both kinds of cell have been observed with the electron microscope.

Gland-cells. Some of the endodermal cells are almost completely filled with large vacuoles, and can be identified as 'cellules spumeuses' or foamy cells (fig. 4, C). Semal (1954*b*) describes them as the most numerous kind of gland-cell, filled with polygonal vacuoles of finely granular content. Their appearances in the light and electron microscopes are very similar and they are thus relatively easy to identify.

Electron micrographs of foamy cells show that the vacuoles appear to be filled with granules, or with a thread-like material, or a mixture of both (fig. 4, C, *vac*), according to their plane of section. At the base of the cell the vacuoles are smaller than those at the periphery and contain a denser granular material. Small mitochondria with internal folds occur infrequently in the tenuous cytoplasm between the peripheral vacuoles, but they become more numerous towards the base of the cell.

Semal (1954*b*) has described the way in which foamy cells discharge at the moment the hydra ingests prey, and then undergo a recharging cycle. Fig. 4, C appears to represent part of a discharging cell. Its vacuoles are similar to those of a resting cell, but they may have altered in size as there seems to be more cytoplasm between them. The mitochondria, of moderate size and with short internal folds, are now easier to discern (fig. 4, C, *mit*). Structures consisting of overlapping or lamellated double membranes may also occur between the vacuoles of a discharging cell, either alone or near mitochondria (fig. 4, C, *lam*).

The discharged material of the foamy gland cells consists not only of vacuoles and their contents but also of portions of the cytoplasm with its mitochondria and lamellated membranes (fig. 4, C). This type of secretion differs from that of the ectodermal gland-cells of the foot, where only secretory granules formed in the cytoplasmic vacuoles leave the cell.

We have not yet observed the presence of muscle-fibres in these cells, nor seen their attachment to the mesogloea.

Digestive cells. Certain other endodermal cells are more variable in structure and probably represent the digestive cells. These are known to undergo profound changes in shape and organization during the digestion of a meal (Semal, 1954*b*). Fig. 4, B shows part of one of these cells filled with relatively

small vacuoles (*vac*): since digestive cells become vacuolated at the beginning of a meal (Semal, 1954b), the state of this cell probably corresponds to an early stage in the digestive process. The peripheral cytoplasm extends into the lumen of the gut as numerous villous processes (fig. 4, B, *vp*), which are covered with external granules, and are a constant feature of these cells during digestion. In addition the digestive cells, like the foamy gland-cells, each possess two flagella (figs. 1, B; 4, B; 5, B; *flag*), as described in the next section. Myofibrils are found in the basal cytoplasm of some digestive cells and are considered further on p. 323.

Generally speaking, the cytoplasm of the digestive cells is light (fig. 1, B) and contains vacuoles of varying sizes, with which granules and mitochondria may be associated. Several other organelles or inclusions may be present. Of these, a round body, whose folded membrane encloses a fairly light substance with complex internal granules, much resembles a similar structure found in ectodermal cells (p. 317, fig. 1, A, *org*).

There is again a complex of membranes associated with vacuoles. Several kinds of inclusions in large vacuoles, smaller vacuoles associated with granules, and dark bodies of variable size whose density is approximately that of lipid material, are among the structures which probably represent stages in the digestion of food products. Similar inclusions have been described by Semal (1954b) and they vary according to the time after a meal and the kind of food ingested.

Flagella. The endodermal cells possess flagella which have been said to number from one to five, with two the most frequent estimate (McConnell, 1931; Mueller, 1950; Semal, 1954b). In our preparations the flagella of both foamy and digestive cells constantly occur in groups of two (figs. 1, B; 5, B, *flag*). Sections of the flagella show the nine peripheral and two central longitudinal filaments which characterize the cilia and flagella of other animals (Fawcett and Porter, 1954). In addition there is a structureless, relatively thick surrounding membrane, which appears to be closely connected to the flagellum but often becomes detached (figs. 1, B; 4, B; 5, B, c). This thick membrane differentiates the flagella of hydra from those of associated organisms. The numerous cilia shown in fig. 5, D, for example, belong to a protozoan situated on a hydra, and lack the thick sheath which surrounds each hydra flagellum. Part of the protozoan is included in the section.

The muscular system

Since the organization of the muscular system in hydra is as yet incompletely known and it is of considerable functional importance, relevant observations made in this study are discussed together below.

Ectodermal muscle. It has been seen that longitudinal sections of the ectoderm show closely packed bundles of fine fibrils, which are orientated longitudinally at the base of the cells and rest on the mesogloea (p. 317; fig. 1, A). The fibrils are spaced evenly and are not cross-striated. Their bundles form a single-layered network extending over most of the animal. In slightly oblique

transverse sections the bundles of fibrils are seen to form a parallel series above the mesogloea (fig. 2, C, *ect*). Since the majority of observers find that the ectodermal muscle-fibres of hydra are also longitudinally orientated, and that they occupy the same positions as the bundles of fibrils we have observed, there is strong indication that the two are identical.

The myofibrils are thus found within epithelio-muscular cells, and they extend right into the long cytoplasmic processes forming the base of each cell (see fig. 6, B). Longitudinal sections sometimes show the muscle-fibres surrounded by basal cytoplasm (fig. 2, A, *ect, bas*), but more often the cytoplasm is so thin that the muscle-fibres seem to rest directly on the limiting membrane of the mesogloea (fig. 3, A, *ect*). It is interesting that the muscle-fibres are not separated from the cytoplasm by a special membrane, although when the cytoplasm in the basal extensions of the cell becomes very thin, the cell-membrane may appear to belong to the muscle-fibre (fig. 3, A). Mitochondria are often found just outside the myofibrils, and they may then bulge from the surface of the muscle-fibre (figs. 2, A, B; 3, A, *mit*).

The muscle-fibres are closely applied to the mesogloea and seem either to push into it themselves (figs. 2, B, C, *ect, mes*), or to be anchored by small pseudopodial processes which penetrate the mesogloea (see *mes* in figs. 2, A, C; 3 A). These appear to be cytoplasmic attachment roots provided by the bases of the epithelio-muscular cells, as described by Semal (1952). Longitudinal sections also show points of contact between the muscle-fibres (fig. 2, C, *j*). Although the membranes of adjacent fibres are intimately connected they remain distinct and the sarcoplasm and fibrils of neighbouring cells do not fuse.

Endodermal muscle. The bases of digestive cells resting on the mesogloea also contain very fine, closely packed fibrils (figs. 2, A, B; 3, A, *end*). They are more difficult to discern than the ectodermal myofibrils owing perhaps to the particular state of contraction of the fixed hydra, but their position and structure indicate that they are of a similar nature and they therefore represent endodermal myofibrils. They are orientated at right angles to those of the ectoderm and form a transverse muscular layer: thus when the ectodermal muscle is cut longitudinally, endodermal muscle is cut in cross-section, and conversely.

All the digestive cells which contain muscle-fibres appear to rest upon the mesogloea. As in the ectoderm, the muscle-containing bases of the cells are in contact with each other, but their respective membranes remain independent, as do their sarcoplasm and fibrils. There is little cytoplasm surrounding the muscle-fibres, however, as they are usually close together and almost fill the base of the cell. While the muscle-fibres are closely connected to the mesogloea, they are smaller and penetrate it less deeply than those of the ectoderm, and cytoplasmic roots of attachment are fewer and less robust than those of the epitheliomuscular cells.

The relation of muscle-fibres to the mesogloea. Frozen-dried sections of anaesthetized hydra stained by Mallory's trichrome method show structures which have been seen by previous authors and also during the present electron

microscope work. The muscle-fibres in particular are very clear. They stain red, as do the muscle-fibres of other coelenterates when coloured by Mallory's method. Transverse sections show that the fibres, situated within the cytoplasm of the epithelial cells, occupy the positions indicated by electron micrographs. Fig. 6, A illustrates this feature in the ectoderm of a contracted specimen in which the muscle-fibres are especially evident. Their position within a single musculo-epithelial cell may be confirmed by comparing this photograph with fig. 6, B, showing an ectodermal cell obtained by the Hertwig osmic-acetic maceration technique (1879). The muscle-fibre lies in the epithelial cytoplasm at the base of the cell, just above the mesogloea, as all recent workers using maceration methods have been able to demonstrate (Goodrich 1942; Mueller, 1950; Semal, 1952). This has been shown in the electron micrographs (figs. 2, A, B, C; 3 A), as described above.

In contrast to the red muscle-fibres the mesogloea stains blue with Mallory's method, and in this respect it resembles the mesogloea of anemones and medusae (Chapman, 1953). The different staining properties of muscle-fibres and mesogloea are illustrated by figs. 6, C, D, which show the same Mallory preparation photographed through blue and red filters. Fig. 6, D shows that the mesogloea forms a distinct layer between the ectoderm and endoderm (see also figs. 2, A, B, C; 3, A; 5, E). It varies in thickness with the state of contraction of the hydra and is best developed in the foot region of this species (*Pelmatohydra oligactis*); Holmes (1950) found a similar distribution in *Chlorohydra viridissima*.

As seen above (p. 323), the electron micrographs show that the muscle-fibres are intimately inserted on to the mesogloea, but that they are separated from it by membranes. Oblique sections in particular show that at least two membranes are present: as well as a membrane limiting the cytoplasm which surrounds each muscle-fibre (p. 323), there is a less clearly defined basement membrane belonging to the mesogloea (fig. 2, C, *doubt*). It has long been known, however, that the muscle-fibres on each side of the mesogloea form two systems of open networks, which are orientated longitudinally, in the ectodermal plane, and transversely, in the endoderm (Mueller, 1950). Between adjacent muscle-fibres, undifferentiated epithelial cytoplasm is therefore in contact with the mesogloea, and other types of cell such as cnidoblasts, or digestive cells without muscle-fibres, may also occupy this position.

It has been suggested by Holmes (1950) that the muscle-fibres represent specializations of the mesogloea and he refers to them as 'mesogloea fibres'. The evidence which has been presented above makes it unlikely that the muscular system of hydra originates from a fluid mesogloea by 'crystallizing out' along lines of force, as has been claimed by Holmes. All types of preparation we have studied show that the muscle-fibres occur within epitheliomuscular cells or digestive cells, and that they belong to these cells and not to the mesogloea. It is difficult also to reconcile Holmes's theory with the observation that distinct membranes are present between the muscle-fibres and mesogloea.

Holmes's explanation (1950) of the organization of the muscular system of hydra, therefore, seems untenable. The problems which it raises, however, as to the nature and origin of the muscle-fibres and mesogloea still remain unsolved. It is to be hoped that future work with the electron microscope will be able to throw light on the much-discussed functional morphology of this small animal.

This paper was written while one of us (A. H.) was on leave of absence at the Anatomy School of Cambridge University, and another of us (E. A. R.) held a D.S.I.R. research assistantship under Dr. C. F. A. Pantin, F.R.S., in the Department of Zoology of Cambridge University. The authors wish to thank Dr. C. F. A. Pantin for his unstinted help and advice in the preparation and production of this paper. The authors are also grateful to Mr. Charles E. Couck, Jr., of the Department of Anatomy of Emory University, Georgia, for his co-operation and technical aid in the production of most of the electron micrographs.

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EXPLANATION OF FIGURES

All the electron micrographs show sections of hydra, and in these the line represents 1μ . Magnifications are approximate.

FIG. 1 (plate). A, section through the ectoderm, showing two epithelio-muscular cells and portions of other cells. The ectodermal surface is to the left.

B, section through the endoderm showing the lumen lined by digestive cells, and flagella in cross-section. Each flagellum is surrounded by a membranous sheath, seen most clearly in the middle pair.

C, section through part of a group of interstitial cells in the ectoderm.

Abbreviations: *dig*, digestive cell; *flag*, flagella; *mf*, myofibrils; *mit*, mitochondria; *nuc*, nucleus; *org*, organelle or inclusion; *surf*, body surface of hydra; *vac*, vacuole; *x*, artifacts in negative.

FIG. 2 (plate). A, B, longitudinal sections through ectodermal muscle-fibres, mesogloea, and endodermal digestive cells with muscle-fibres in their bases.

C, oblique section through ectodermal muscle-fibres and mesogloea.

Abbreviations: *bas*, basal cytoplasm of epithelio-muscular cell; *doub*, double membrane between muscle-fibre and mesogloea; *dig*, digestive cell; *ect*, ectodermal muscle; *end*, endodermal muscle; *j*, junction of muscle fibres; *mes*, mesogloea.

FIG. 3 (plate). A, longitudinal section through ectodermal muscle-fibre, mesogloea, and endodermal digestive cells with muscle-fibres in their bases. The large space above the longitudinal muscle-fibre is an artifact produced by the tearing away of the muscle from the ectoderm.

B, section through an undischarged stenotele.

C, section through portion of an undischarged holotrichous isorhiza, showing the overlapping layers of electron-dense spines.

D, section through part of an undischarged stenotele, showing overlapping layers of electron-dense barbs within the serrated outline of the butt wall.

Abbreviations: *b*, butt of stenotele; *dig*, digestive cell; *ect*, ectodermal muscle; *end*, endodermal muscle; *mes*, mesogloea; *mit*, mitochondrion; *oc*, outer capsule of nematocyst; *s*, stylet of stenotele; *sp*, spines of holotrichous isorhiza; *t*, tube of stenotele.

FIG. 4 (plate). A, section through the ectoderm showing a group of cnidoblasts with immature nematocysts.

B, section through part of a digestive cell at an early stage in the digestive process. The flagellum is surrounded by a closely applied membranous sheath.

C, section through portion of a discharging foamy gland-cell. Part of the same cell is seen at the bottom left-hand corner of the previous photograph, at higher magnification.

Abbreviations: *al*, amorphous layer; *con*, regions of apparent cytoplasmic continuity between adjacent cells; *flag*, flagella; *lam*, lamellated membranes; *mit*, mitochondria; *n*, nucleolus; *nem*, immature nematocyst; *nuc*, nucleus; *vac*, vacuole; *vp*, villous projection.

FIG. 5 (plate). A, section through ectodermal gland-cells of the pedal disk.

B, section through endoderm showing paired flagella belonging to a foamy gland-cell and to a digestive cell.

C, longitudinal section of a flagellum from an endodermal gland-cell.

D, section through part of a ciliate protozoon found attached to hydra.

E, oblique section showing fibrous mesogloea between ectoderm and endoderm.

F, peripheral part of two ectodermal cells to show the surface.

Abbreviations: *al*, amorphous layer; *ect*, ectoderm; *end*, endoderm; *flag*, flagella; *gr*, granules; *mes*, mesogloea; *org*, organelle or inclusion; *sh*, surrounding membrane of flagellum; *surf*, body surface of hydra; *vac*, vacuole.

FIG. 6 (plate). Light microscope photographs of *Pelmatohydra oligactis* material. All except B are of frozen-dried sections, treated with Baker's formaldehyde-calcium and stained with Mallory.

A, transverse section showing the row of longitudinal muscle-fibres in the body-wall. They lie within the ectoderm cells and above the mesogloea. The lower half of the figure contains endoderm.

B, musculo-epithelial cell from the ectoderm (osmic-acetic maceration / picrocarmine) showing a straplike muscle-fibril within the basal cytoplasm. Note the large nucleus.

C, transverse section of the body-wall, with ectoderm above and endoderm below. The photograph is taken with a blue filter (Wratten C₅+H) to bring out structures which have been stained red. The longitudinal and circular muscle-fibres show darkly on each side of the mesogloea, which is pale.

D, the same section, photographed with a red filter (Wratten A). The mesogloea is now dark since it is stained blue, and the muscle-fibres do not show at all.

E, vertical section through the foot, showing the ectodermal gland-cells. Note distal granules, compact cytoplasm, and basal muscle-fibres.

F, tangential section through distal part of foot ectoderm, showing the distribution of secretory granules.

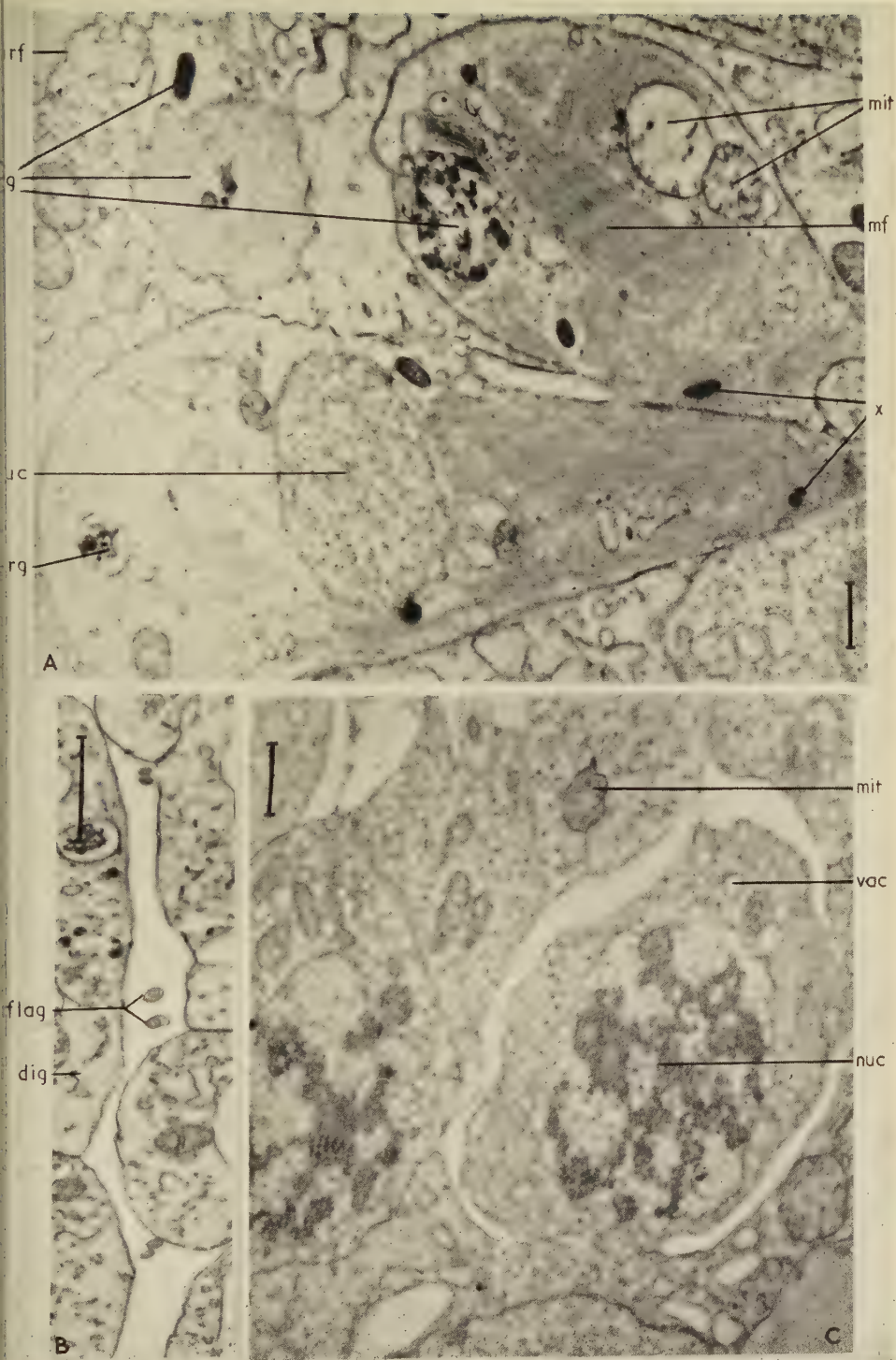


FIG. 1

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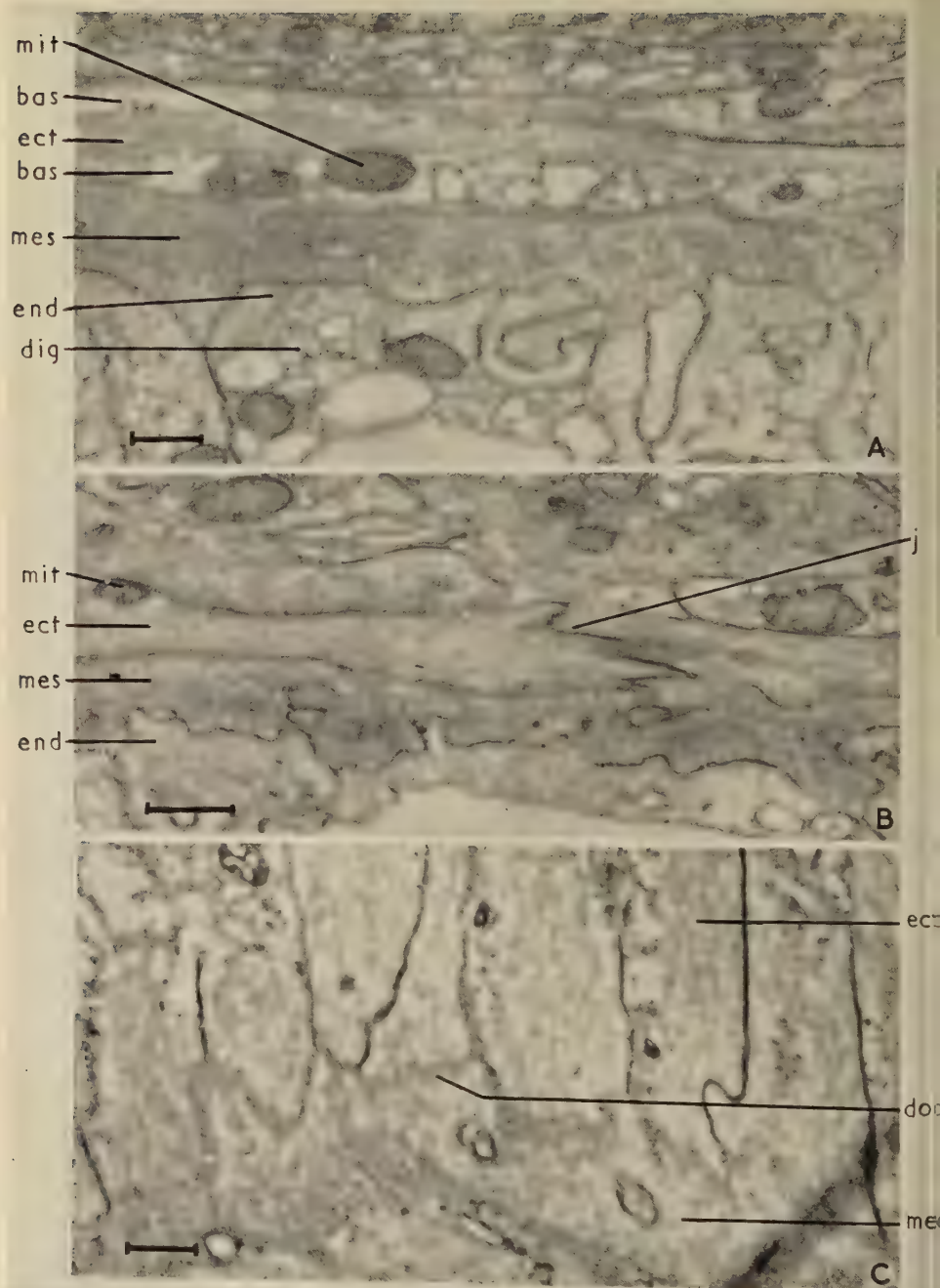


FIG. 2

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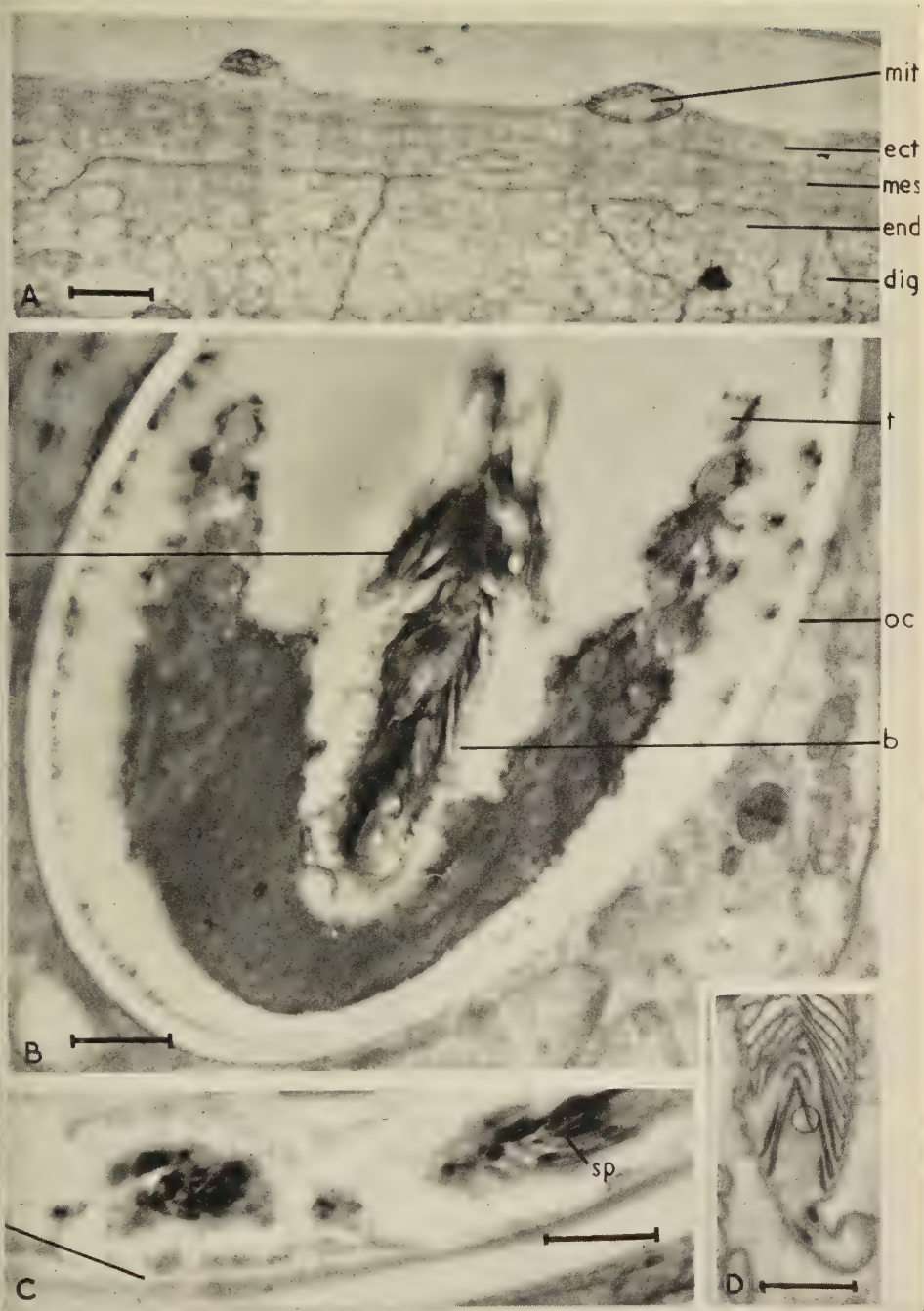


FIG. 3

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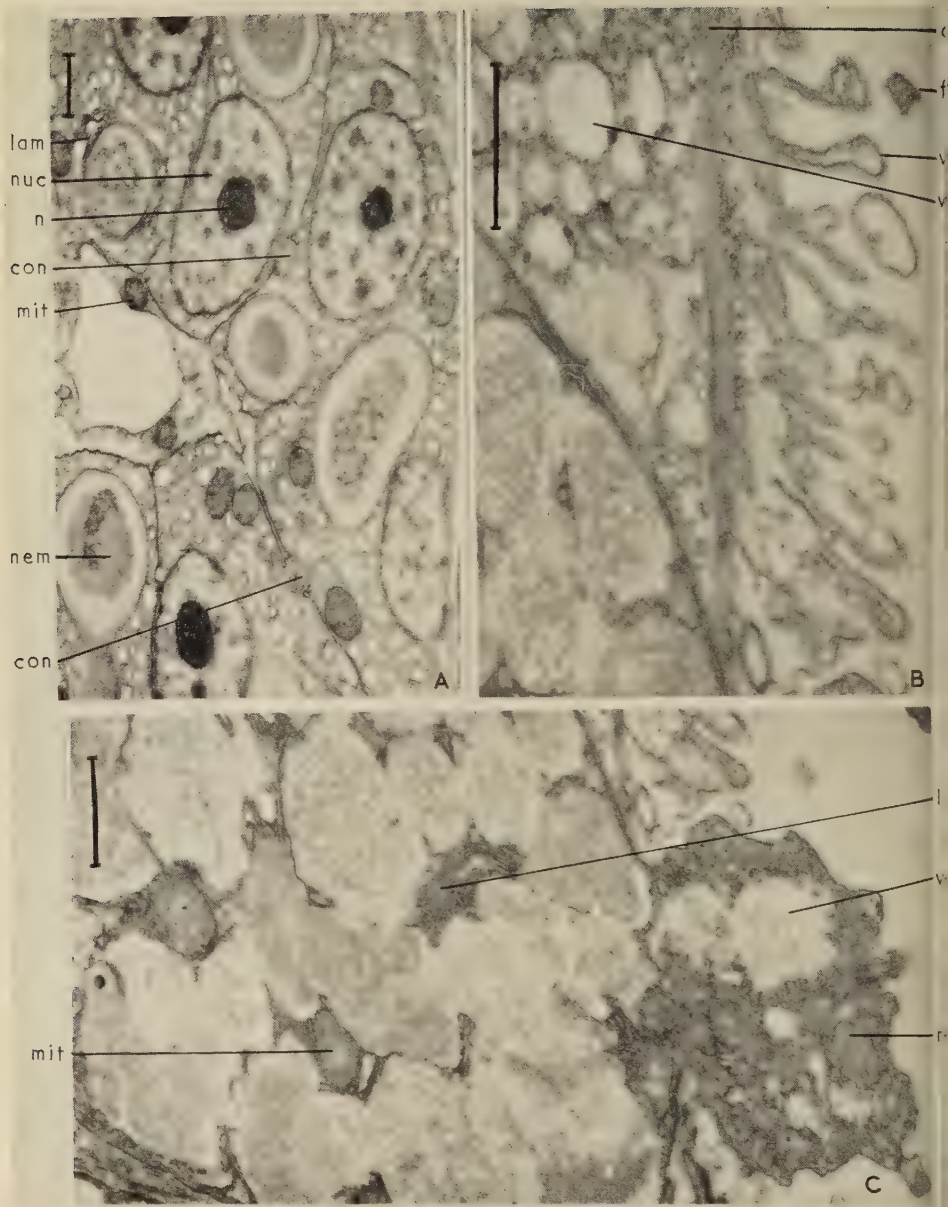


FIG. 4

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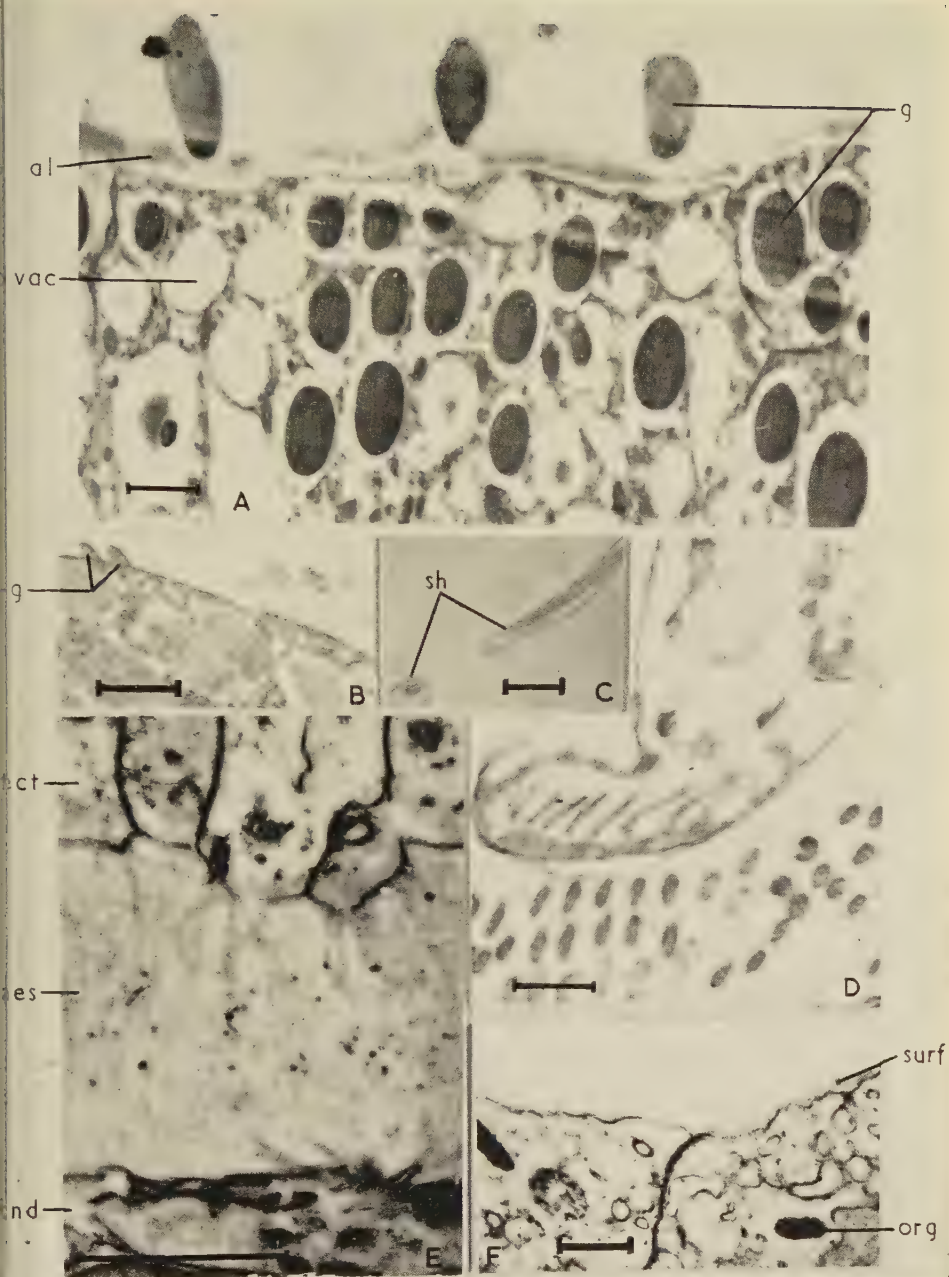


FIG. 5

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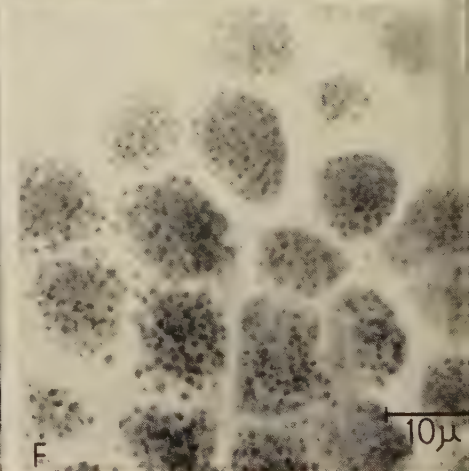
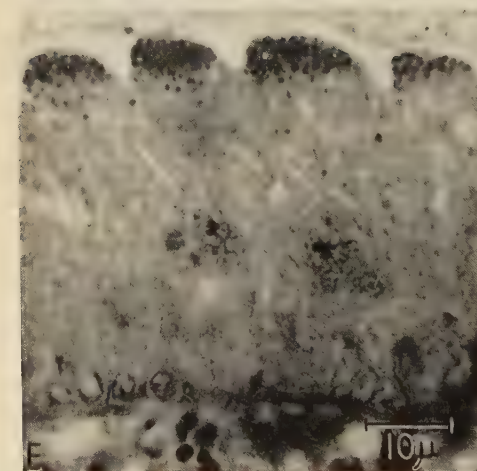
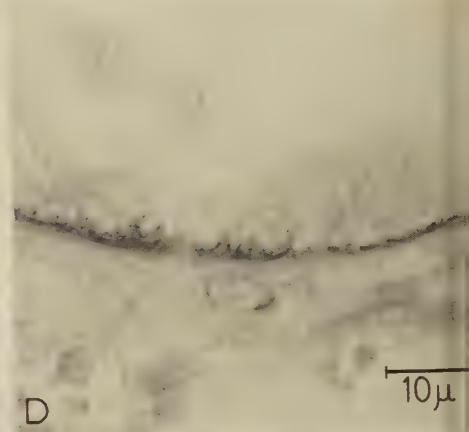
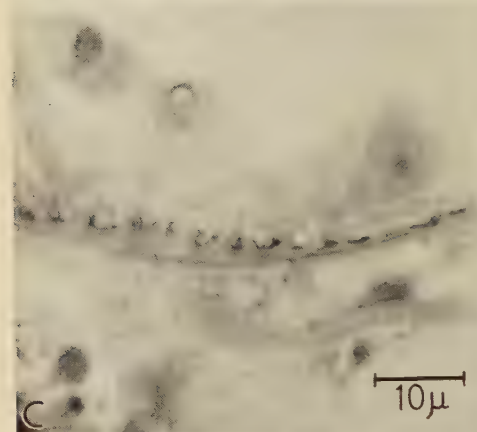
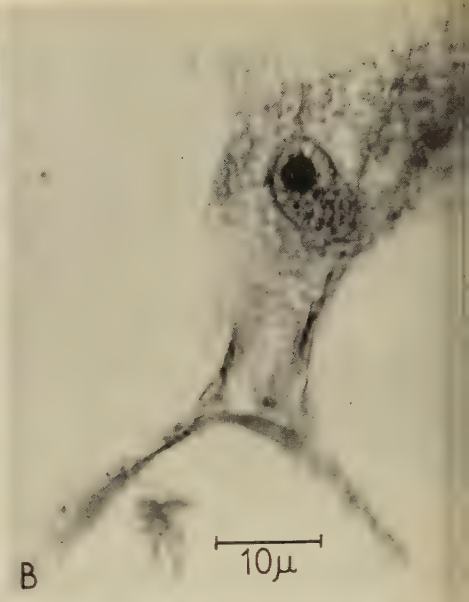
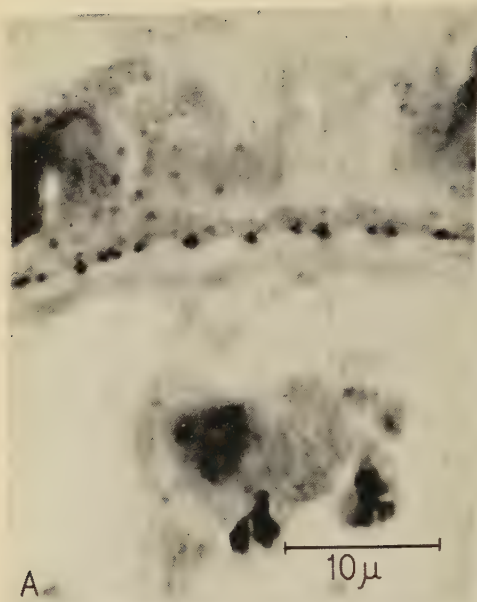


FIG. 6

A. HESS, A. I. COHEN, and E. A. ROBSON

The use of Cholinesterase Techniques for the Demonstration of Peripheral Nervous Structures

By R. E. COUPLAND AND R. L. HOLMES

(From the Department of Anatomy, University of Leeds)

With two plates (figs. 1 and 2)

SUMMARY

An account is given of a modification of the method of Koelle and Friedenwald for the demonstration of cholinesterase activity. When this method is employed, diffusion artifacts are minimal, and good histological pictures of central and peripheral nervous structures can be obtained. Certain advantages of this method over metallic impregnation and methylene blue techniques for demonstration of nervous elements are discussed, and its application to a variety of tissues described.

INTRODUCTION

INVESTIGATIONS into the fine structure of the peripheral nervous system have depended to a great extent on metallic impregnation techniques, especially those involving the use of silver. A large number of variations have been described (see Addison, 1950). Although these methods are extremely valuable, they have several disadvantages, of which the worst are inconsistency of results and a tendency for the impregnation of non-nervous elements. Bielschowsky techniques are especially prone to the latter fault, and results may be particularly misleading when the tissue is rich in reticular fibres, which are easily confused with nerves. Silver impregnation techniques also suffer from the disadvantage that relatively thin sections are essential; this enables impregnated structures to be examined at high magnifications, but hinders the easy perception of the inter-relationship of various elements. Ehrlich's methylene blue method and its later modifications have also been widely used for nerve staining. Recently Hillarp (1946), Meyling (1953), and Mitchell (1956) have made wide use of this dye in investigations into the fine structure of the peripheral extensions of the autonomic nervous system. The tissue used for this method of staining must be fresh and unfixed. It is preferable, indeed, to inject the dye while the animal is still living (Mitchell, 1953). Although satisfactory demonstration of nerve-fibres in many organs may be obtained by the use of this dye, the difficulty of obtaining adequate sections, while at the same time retaining the stain, limits the use of the method. Whole mounts of suitable tissues, such as heart-wall (Holmes, 1957) may, however, be made.

METHODS AND APPLICATIONS

The cholinesterase technique was introduced by Koelle and Friedenwald (1949) and improved by Koelle (1951). It has subsequently been modified by Coërs (1953), Snell and McIntyre (1956), and by Coupland and Holmes

(1957). The method has been found to be of value in the demonstration of nervous elements in many different situations. We have found that the technique which gives the best results with mammals is as follows.

Tissues are removed from the animal as soon after death as possible although adequate preparations have been obtained with post-mortem material received 8 h after death. The tissues are fixed in 10% formol-saline for 14–24 h at about 4° C. Frozen sections of the required thickness are then cut and washed in isotonic saline for 30 min or longer. These are mounted on clean slides and allowed to dry in air for about 20 min to ensure adherence. The slides are then incubated in the following solution:

copper glycine 0.6 ml (glycine, 3.75 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2.5 g; distilled water to 100 ml)

magnesium chloride solution, 0.6 ml ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 9.52 g; distilled water to 100 ml)

M/5 acetic acid-sodium acetate buffer at required pH, 5 ml

sodium sulphate solution, 7.6 ml (Na_2SO_4 , anhydrous, 40 g; distilled water to 100 ml)

acetyl thiocholine or butyryl thiocholine solution, 1.2 ml (acetyl thiocholine or butyryl thiocholine iodide, 23 mg; distilled water, 1.2 ml; CuSCN (0.1 M solution), 0.4 ml; centrifuged, and supernatant decanted for use)

After incubation the tissues are washed for 2 min in water, treated with dilute ammonium sulphide, washed again in water for 2 min, dehydrated and mounted in Canada balsam. Slides should be allowed to dry after mounting at room temperature. A fine brown deposit is formed at sites of cholinesterase activity. If this method is followed, diffusion artifacts are minimal.

The pH of the solution is of vital importance in the demonstration of nerve fibres. The optimum pH varies with species, while the incubation time is directly dependent upon it. The use of a pH of 6 and above results in a heavy background deposit due to the presence of pseudocholinesterase and non-specific esterases. As a result of this, nerve-fibres, although giving a positive reaction, are not readily observed. It is therefore necessary to use a pH which is compatible with a satisfactory reaction in the nervous elements, but which gives minimal general tissue reaction. This has been found to be pH 5.6 in the rabbit, pH 5 in the rat, pH 4.6–5 in man, cat, and dog. The use of a low

FIG. 1 (plate). A, part of nerve network in atrial wall of rabbit heart. 200- μ section, acetyl thiocholine substrate, pH 5.6, incubation time 16 h.

B, thick nerve-fibres giving off fine branches which form a net around a small vessel running across the lower part of the figure. 200- μ section rabbit atrial wall, butyryl thiocholine substrate, pH 5.6, incubated 16 h.

C, Pacinian corpuscle in cat pancreas. A positive reaction occurs over the central core. A nerve-net may be seen in the surrounding acinar tissue. Acetyl thiocholine substrate, pH 5, incubation time 5 h.

D, nerve-net in cornea of rat. Whole mount. Acetyl thiocholine substrate, pH 5, incubation time 16 h.

E, Auerbach's plexus in cat ileum. Acetyl thiocholine substrate, pH 5, incubation time 6 h.

F, Meissner's plexus in rabbit ileum. Acetyl thiocholine substrate, pH 5.6, incubation time 16 h.

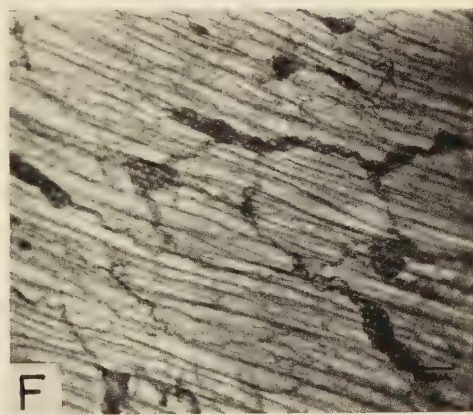
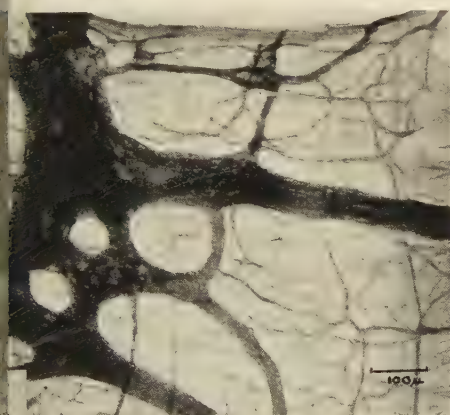
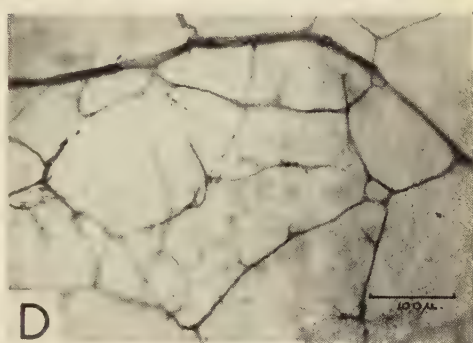
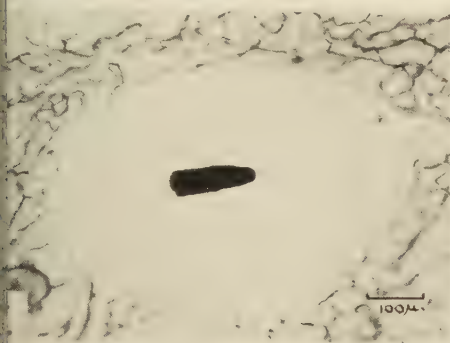
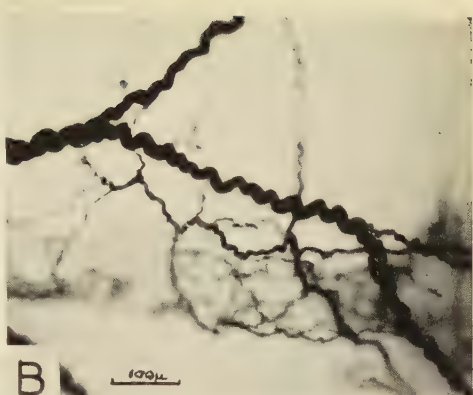
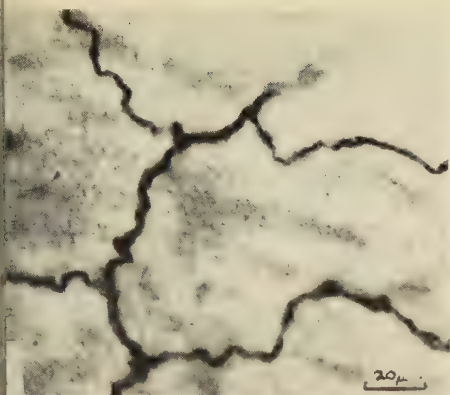


FIG. 1

R. E. COUPLAND and R. L. HOLMES

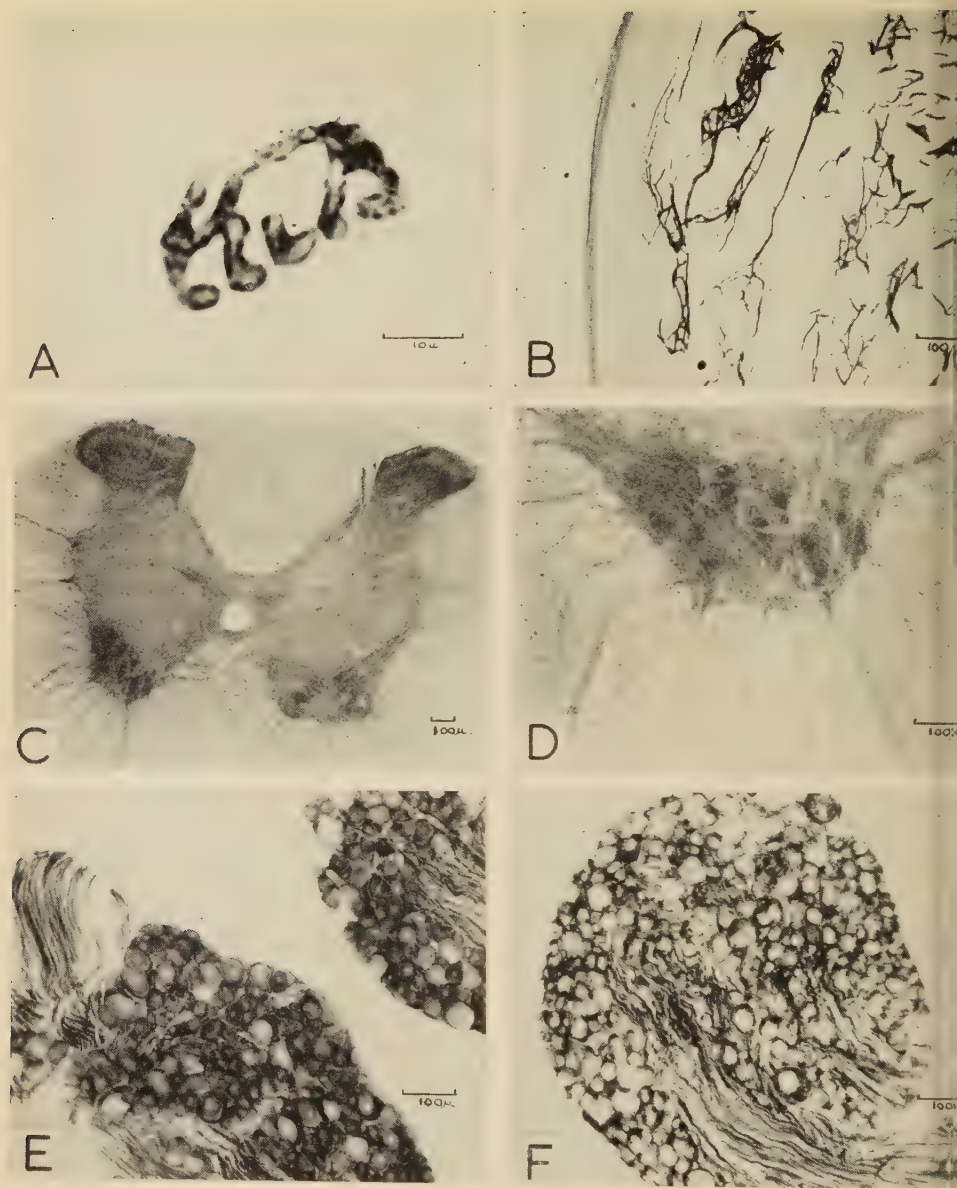


FIG. 2

R. E. COUPLAND and R. L. HOLMES

pH as employed by Snell and McIntyre (1956) and Snell and Garrett (1956), though of value in demonstrating sites of maximum cholinesterase activity and differentiating pre- and post-ganglionic sympathetic nerve-fibres, produces a very incomplete picture of the entire nerve-net in an organ, and as reported by Bergner (1957) fails to show small accumulations of cholinesterase in denervated motor end-plates.

The cholinesterase enzymes are usually divided into two distinct groups, true cholinesterases and pseudocholinesterases (Hawkins and Mendel, 1947). These may be distinguished histochemically by the use of di-isopropylfluorophosphate (DFP). After the tissue sections have been mounted on slides, they are immersed for 20 min in a 10^{-6} or 10^{-7} M solution of DFP in saline, rinsed for 5 min in two changes of saline, and finally placed in the incubating solution. Incubation of sections treated with DFP in a substrate containing acetyl thiocholine gives a positive reaction for true cholinesterase activity and usually gives a good demonstration of the nerve plexus; while incubation with butyryl thiocholine at the optimum pH for the species usually gives a negative result.

All nerve-fibres examined so far in man, cat, dog, rat, and rabbit have been found to contain both true and pseudocholinesterase. In foetal material most of the activity is due to pseudocholinesterase, and hence butyryl thiocholine is the most suitable substrate. In most post-natal tissue true and pseudocholinesterase are present in more or less equal amounts, and acetyl thiocholine is usually the most useful substrate, as the reaction due to tissue pseudocholinesterase is reduced to a minimum. Sensory nervous structures in the heart-wall of the cat and dog do, however, give a much stronger reaction with butyryl thiocholine.

Pseudocholinesterase activity is not confined to nervous elements, but may also be found, for example, in smooth muscle or capillary walls of the nervous system (Koelle, 1954). In the dog pancreas and adrenal the concentration of pseudocholinesterase in the capillary vessels and in acinar tissue has been found to be so great that in order to observe nerve-fibres tissues must be pre-treated with 10^{-6} M DFP even when acetyl thiocholine is used as a substrate.

By comparison with other methods the cholinesterase technique has several

FIG. 2 (plate). A, motor end-plate in rat diaphragm. Acetyl thiocholine substrate, pH 5, incubation time 20 min.

B, cervical region of rat uterus showing nerve-net in association with myometrium and vessels. Endometrium lies on the left. Acetyl thiocholine substrate, pH 5, incubation time 20 h.

C, thoracic region of rat spinal cord. A positive reaction occurs in nerve-cells of the dorsal, ventral, and lateral horns and in nerve-fibres. Section pre-treated with 10^{-6} M DFP, acetyl thiocholine substrate, pH 5, incubation time 16 h.

D, anterior horn of rat spinal cord shown in the previous figure (1). A positive reaction is given by all except the nuclear region of the anterior horn-cells.

E, dorsal root ganglion of rat. A positive reaction, due to true cholinesterase, is given by all except the nucleus of the cell-body, by satellite cells, and nerve-fibres. Acetyl thiocholine substrate, pH 5, incubation time 16 h.

F, dorsal root ganglion of rat. A positive reaction, due to pseudocholinesterase, is limited to the satellite cells and nerve-fibres. Butyryl thiocholine substrate, pH 5, incubation time 16 h.

advantages in the demonstration of nervous structures. The method is relatively simple to perform and has been found to be reliable. The combination of preliminary formalin fixation, buffering the substrate to an acid pH, and inclusion of sodium sulphate reduce diffusion artifacts. Also by ensuring a finely divided final precipitate, good microscopic appearances are obtained even with high magnifications (fig. 1, A). Although the method is not absolutely specific for nerve-fibres, the use of two substrates combined with suitable inhibitors can obviate any possible confusion between nerve-fibres and other structures. Appearances equal to those found in good methylene blue preparations have been obtained. The technique is particularly suitable for demonstrating the nerve-fibres which occur in the walls of hollow viscera or around blood-vessels. Nerve-fibres have been shown in thin sections of adrenal gland (Coupland and Holmes, 1957) and pancreas (Coupland, 1957), and the method has been found especially useful in the study of nerves of the mammalian heart (fig. 1, B). Receptor organs such as Pacinian and corpuscles and free nerve-fibres in the cornea (fig. 1, C, D) are well shown. Other structures which have been demonstrated include Auerbach's and Meissner's plexuses, and motor end-plates and nerves in the uterine wall (figs. 1, E, F; 2, A, B). Sections of spinal cord and ganglia also give good results (fig. 2, C-F).

The preliminary fixation in formalin, which is not possible with methylene blue techniques, allows easy cutting of satisfactory frozen sections. Nerves can be demonstrated readily in sections 10 to 200 μ in thickness, or in whole mounts of the walls of thin-walled viscera. The latter preparations present a complete picture in depth of the nervous elements in a single preparation. This is rarely possible with metallic impregnation methods. The sections appear to be permanent, although slight diffusion may occur in the balsam, particularly if the slides are dried in the oven.

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Spider Leg-muscles and the Autotomy Mechanism

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SUMMARY

Spider leg-muscles have been redescribed and the absence of extensor muscles at the joints confirmed. The possibility that the flexors at these joints act in antagonism to the blood-pressure is discussed. The coxal muscles are inserted on to sclerites in the trochanter and autotomy occurs at this joint. At autotomy the sclerites are detached from the trochanter and support the articular membrane, which restricts flexing.

INTRODUCTION

PETRUNKEVITCH described the leg-muscles of spiders in 1909, correcting earlier and very formalized conceptions and drawing attention to the absence of extensor muscles at several of the joints. Brown (1939) and Ellis (1944) agreed with Petrunkevitch's account, and Ellis considered the role of blood-pressure in extending the joints which lacked extensors, suggesting that certain muscles operated valves to produce a local rise of pressure. Dillon (1952) redescribed the leg musculature: he maintained that extensors were present at all but one, or possibly two, joints and differed in other ways from Petrunkevitch. Snodgrass (1952) modified Dillon's account of the lower leg, omitting details of the coxal muscles. The families of spiders examined by these authors were:

Petrunkevitch:	Theraphosidae, Lycosidae, Eusparassidae.
Brown:	Agelenidae.
Ellis:	Theraphosidae, Agriopidae, Theradiidae, Agelenidae.
Dillon:	Theraphosidae, Agriopidae.
Snodgrass:	Theraphosidae.

None of the authors who examined more than one family reported any significant differences between them, and as Petrunkevitch, Dillon, and Snodgrass all included the large *Tarantula Eurypelma* sp. (Theraphosidae), the differences in their accounts appear to be those of interpretation and not of systematics.

This paper contains an account of the leg-muscles of the common British house spider *Tegenaria atrica* (Koch: Agelenidae). I have followed Snodgrass (1952) and recent entomological practice in referring to the muscles by number rather than by means of the earlier functional nomenclature which leads to difficulties when function is in doubt or when comparative studies indicate that muscles differing in function are nevertheless homologous. The corresponding muscles in the accounts of Petrunkevitch, Dillon, and Snodgrass have been indicated as far as possible, the abbreviations P, D, and S being

used for this purpose; but a full appreciation of the similarities and difference requires a comparison of the drawings.

A close examination of the coxal muscles has brought to light further facts about the autotomy mechanism of spiders, first described by Bonnet (1930). Unlike crustacea and insects, spiders autotomize their legs at a functional joint and an account is given of the interesting mechanism by which the joint is severed and bleeding restricted.

The musculature has been worked out by ordinary dissection of adult animals under a binocular microscope. Although the account refers specifically to *T. atrica*, I have had the advantage of making a preliminary dissection of a specimen of the much larger *Lycosa ingens* (Blackwall: Lycosidae) from Deserta Grande (Madeira) kindly given to me by Mr. Roderick Fisher. Only minor differences appear to exist between the two species.

THE ARTICULATION AND MUSCULATURE OF THE LEGS

The leg-muscles and articulations of *Tegenaria atrica* are shown in figs. 2 and 3. Each part of the limb is shown as though examined by transparency from a lateral face as far as the median vertical plane. The coxa, trochanter, femur, and patella are viewed from both anterior and posterior faces, but the tibia, basitarsus, and telotarsus are seen only from one face as the musculature is symmetrical about the median vertical plane. The coxa is also drawn as seen from above, both from the dorsal surface as far as the median horizontal plane (fig. 3, C) and from this plane down to the ventral surface (fig. 3, D). It is not proposed to repeat in the text what is evident in the diagrams but to discuss the probable functions of the muscles, considering movements about each joint in turn, starting at the distal end of the leg.

Claw (fig. 1, D). Like most web-spinning spiders the Agelenidae have three claws, paired dorsal ones and a smaller median one. The paired claws are attached to the thin cuticle at the end of the telotarsus and have considerable freedom of movement. The median claw is an extension of a sclerotized terminal plate (the pretarsus of Snodgrass) and is relatively rigid on the telotarsus. To the extreme dorsal and ventral points of the terminal plate are attached the long median tendons of the median muscle 27 which elevates the claws, and the paired muscles 28 and 29 which depress them.

Muscle 27 (median) = M. extensor unguium (P) = M. levator pretarsi (D) = 21 (S—fig. 25)

Muscles 28 and 29 (paired) = M. flexor unguium (P) = M. depressor pretarsi (D) = 22 (S—fig. 25)

I agree with Snodgrass that muscles 28 and 29 do not have the origins in the basitarsus figured by Petrunkevitch and Dillon.

Basitarsus-telotarsus joint (fig. 1, D). There are no muscles directly associated with this joint, a condition commonly found at joints subdividing the tarsus of insects. Nevertheless, the joint plays a part in leg movement and owing to the disposition of the long tendon of muscles 28 and 29, contraction of these

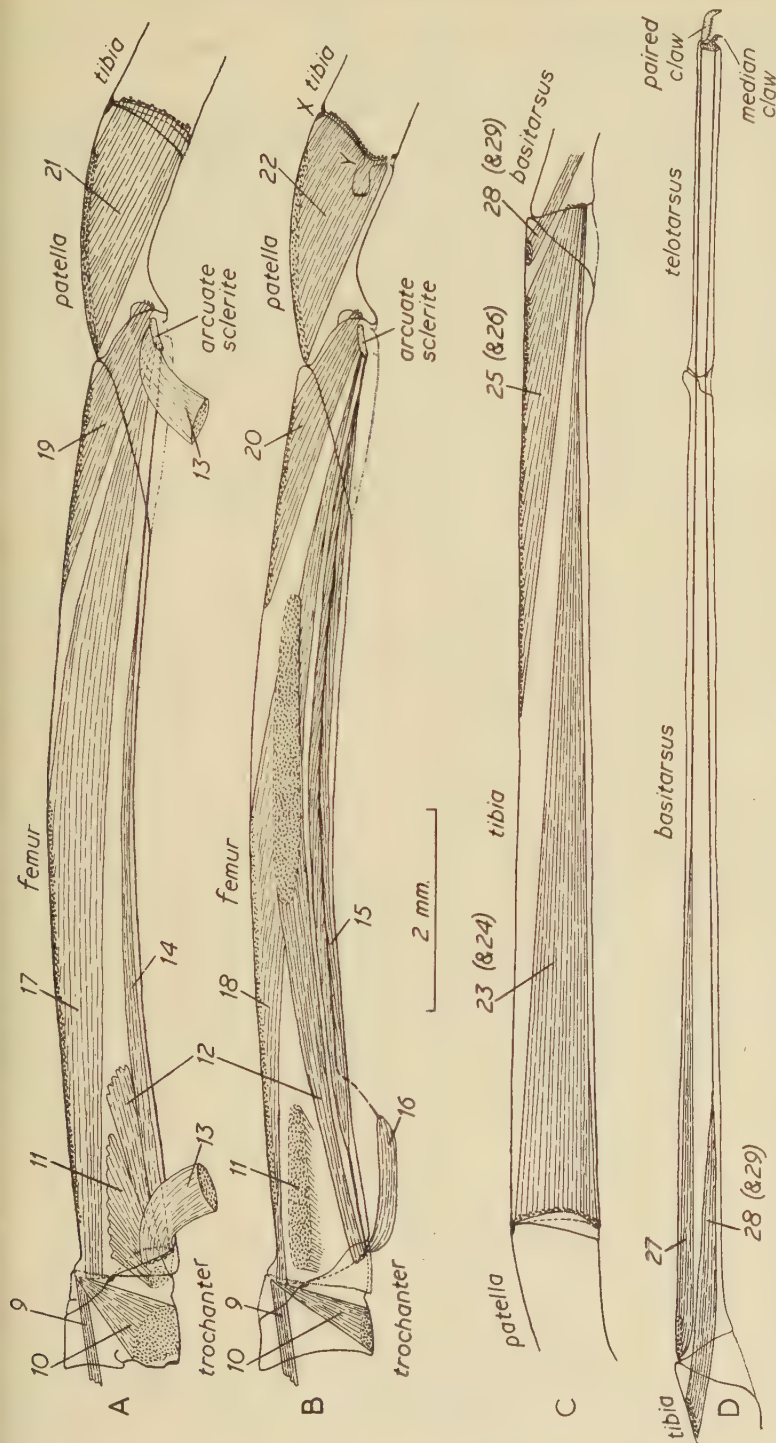


FIG. 1. Leg-muscles of *Tegenaria atrica*. A, trochanter, femur, and patella seen from the anterior face. B, the same, from posterior face. C and D, tibia, basitarsus, and telotarsus seen from the anterior face. Apart from the patella-tibia articulation, the view from the posterior face is similar, but note that the claw tendons are median.

depresses the telotarsus as well as the claws. Contraction of muscle 27 appears to have little effect. There is no articular condyle at the joint which consequently is a universal one.

Tibia-basitarsus joint (fig. 1, c). The articulation is along a dorsal hinge line, so the possibility of muscular elevation of the basitarsus is excluded. Laterally and ventrally the articular membrane is extensive, folding up during depression and becoming extended and slightly convex during elevation. Depression is due to two pairs of muscles 23 and 24, and 25 and 26. Although muscles 28 and 29 originate at the distal end of the tibia, they do so close to the hinge line, and have no significant effect at this joint.

Muscles 23 and 24 (paired) = M. flexor metatarsi longus (paired) (P)
= M. flexor tarsi anticus and posticus (D)
= 19 and 20 (S—fig. 25)

Muscles 25 and 26 (paired) = M. flexor metatarsi bilobatus (paired) (P)
= M. flexor tarsi major and minor (D)
= 17 and 18 (S—fig. 25)

Petrunkévitch figures some fibres of the paired muscles 23 and 24 originating laterally at the anterior end of the patella: this has not been confirmed by Dillon, Snodgrass, or myself. I have, however, found a small slip of muscle 23 (the anterior one) which extends proximally a short way into the patella where it appears to terminate on a nerve-fibre and may be proprioceptive in function. This unusual arrangement will be dealt with in a later paper.

Patella-tibia joint (fig 1, A, B). There is a single dorsal condyle at X and the joint is free to move in a horizontal plane only, being capable of more retraction than protraction. This movement is due to the two muscles 21 and 22 which lie wholly within the patella. The posterior rim of the patella is indented as shown at Y in fig. 1, B, and the extent of the articular membrane in this region varies in the different legs, being least in the hinder two legs (shown in fig. 1, B) and more extensive in the anterior two legs. Movement at the joint causes marked distortion in the indented region of the patella rim and it is thus significant that at the head of the indentation lie two lyriform organs (with their axes at right angles) which Pringle (1955) has shown to be proprioceptors.

Muscles 21 and 22 (paired) = M. promotor and retractor tibiae (P)
= M. promotor and remotor tibiae (D)
= 14 and 15 (S—fig. 25)

In addition to this pair the other authors have described other muscles in the patella, the presence of which I do not confirm. Petrunkevitch figures M. flexor tibiae originating dorsally at the proximal end of the patella and inserting by a tendon ventrally at the proximal end of the tibia, which he says is 'to all appearances functionless'. Snodgrass figures a ventral muscle (muscle 16, fig. 25) with an undescribed origin and anteroventral insertion. Dillon figures M. depressor tibiae anticus and posticus originating dorsally

the distal end of the femur and inserting ventrally, at the proximal end of the tibia in the Theraphosidae, and on the side of the patella in the Agriopidae. Having examined many serial sections of the femuro-patella joint I am sure that this muscle does not exist in *Tegenaria*. I also do not confirm that Dillon's M. promotor tibiae (my muscle 21) has the complex form that he illustrates.

Femur-patella joint (fig. 1, A, B). The articulation is along a dorsal hinge-line with condyles at each end, so the possibility of elevator muscles is precluded (as at the tibia-basitarsus joint). Owing to the swept-back form of the distal rim of the femur, there is a very large area of articular membrane laterally and ventrally. This becomes folded when the joint is flexed (fig. 2)

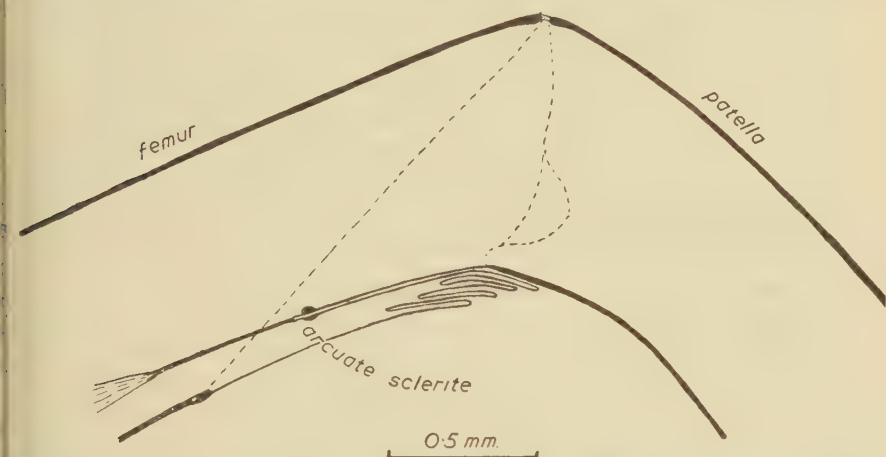


FIG. 2. *Tegenaria atrica*: vertical median section through the flexed femur-patella joint, showing the folded articular membrane. Note that the arcuate sclerite is formed as a thickening and sclerotization of a permanent (crescent-shaped) fold of the membrane.

and slightly convex when the joint is fully extended. Mid-ventrally near the patella there is a semicircular invagination of this membrane the rim of which is sclerotized to form an arcuate sclerite, convex proximally (see figs. 1 and 2). This sclerite is referred to by Ellis as the chitinous plate, and by Dillon as the myophoric lamella.

The muscles originating within the femur and producing depression (flexion) are:

Muscles 17 and 18 (paired) originating along the mid-dorsal line over the proximal half of the femur and inserted on to the arcuate sclerite.

Muscles 19 and 20 (paired) originating along the mid-dorsal line over the distal quarter of the femur and inserted on to the sclerite and the rim of the patella.

Muscle 13 originating on the antero-ventral proximal rim of the femur and inserted on to the anterior half of the sclerite.

In addition to the above muscles there is a muscle complex composed of

muscles 14, 15, and 16 which originates on the ventral rim of the trochanter and is inserted through two tendons on the arcuate sclerite, thus having depressor action at both trochanter-femur and femur-patella joints. The more anterior muscle 14 is almost distinct from the rest of the complex, but does receive a few fibres (not shown in fig. 1) from the more posterior muscle 15. Muscle 15 originates on a tendon from the trochanter and the fibres insert along the length of another tendon which runs to the arcuate sclerite. A distinct fibre group, muscle 16, originates on the trochanter lip and inserts on the same tendon as the fibres of muscle 15.

Muscles 17 and 18 (paired) = *M. flexor patellae bilobatus* (paired) (P)
 = *M. flexor patellae robustus* (single) (D)
 = 9 and 10 (S—fig. 25)

Muscles 19 and 20 (paired) = *M. flexor patellae bilobatus* (paired) (P)
 = *M. flexor patellae major* (anterior) and
minor (posterior) (D)
 It is not figured by Snodgrass.

Muscle 13 is considered by Petrunkevitch to be part of the *M. flexor patellae longus* to which he gives a double origin. It may correspond to Dillon's *M. flexor patellae brevis*. It is not figured by Snodgrass.

Muscles 14, 15, and 16. These are represented by Petrunkevitch as a single muscle, his *M. flexor patella longus*. They probably correspond to the distinct muscles named by Dillon *M. extensor femoris proximalis*, *princeps*, and *communis* respectively. They also appear to correspond to the two lateral and one ventral muscle figured by Snodgrass (his muscles 11, 12, and 13, fig. 25).

Trochanter-femur joint (fig. 1, A, B). This is the only dicondylar joint, the condyles being lateral so that movement is limited to the vertical plane. Depression is due to muscles 11 and 12 lying in the femur and elevation to muscle 10 in the trochanter and to a smaller muscle (9) originating in the coxa.

Muscles 11 and 12 = *M. extensor femoris posticus* (D)

They are not figured by Petrunkevitch. Snodgrass figures a single muscle (his muscle 8, fig. 25) which is inserted dorsally along the anterior half of the femur and most nearly corresponds to my muscle 11.

Muscle 10 = *M. flexor femoris bilobatus* (paired) (P)
 = *M. flexor femoris bilobatus* and *dubius* (D)
 = 7 (S—fig. 25)

Muscle 9 = *M. flexor femoris longus* (P)
 = *M. gracilis* (D)
 = 6 (S—fig. 25)

Both Dillon and Snodgrass figure a slip of this muscle originating on the anterior rim of the trochanter, which I do not confirm.

Coxa-trochanter joint (fig. 3, A-D). This involves the only ball-and-socket

joint in the leg, the socket being formed in the anterior rim of the trochanter (fig. 1, A, C); and the 'ball' being the enlarged end of a long horizontal apodeme which projects into the coxa along the mid-lateral line on the anterior face. There is no other condyle and the joint is free in the vertical and horizontal plane. Fitting closely into a groove in the rim of the trochanter are five slender sclerites (fig. 3, a-e), while a sixth sclerite runs obliquely across the articular membrane on the posterior face of the joint (fig. 3, B, E, f). All the eight coxal

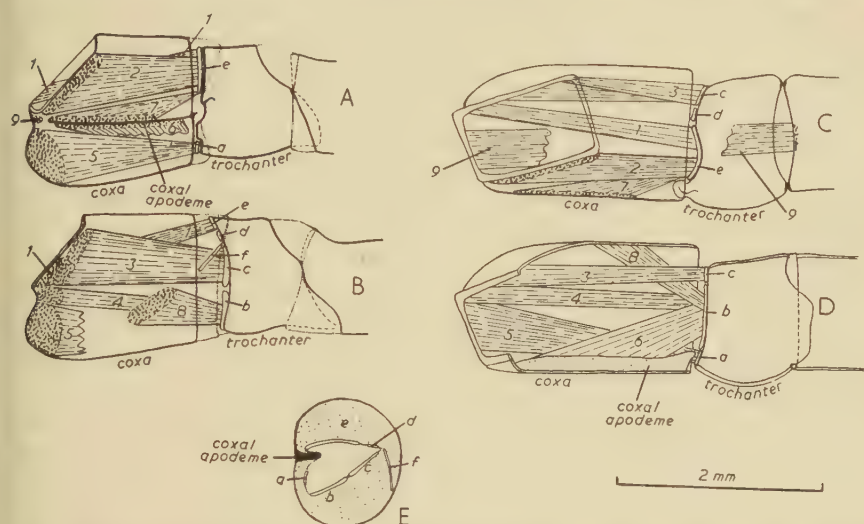


FIG. 3. *Tegenaria atrica*: muscles of the coxa and trochanter. A and B, anterior and posterior faces respectively. C, dorsal view extending down to the level of the coxal apodeme, which, for clarity, is not drawn but lies just below muscle 7. D, view extending down from the level of the coxal apodeme to the ventral surface. E, distal end of the coxa immediately after autotomy.

muscles are inserted on to these sclerites and not on to the trochanter itself: their function will be discussed below. Muscle 9, which passes through the joint to insert on the femur, has already been described. No attempt has been made to analyse the functions of the coxal muscles and it is unlikely that they act singly. Petrunkevitch gives a very simplified account of the coxal musculature describing only four muscles (*M. flexor*, *extensor*, *promotor*, and *retractor trochanteris*). Similarly Snodgrass refers to only five major groups of fibres, two being dorsal and three ventral, which he does not figure in any detail. Dillon figures nine muscles besides the one inserted on to the femur (my muscle 9), seven of which are approximately related to those shown in my fig. 3 as follows:

- Muscle 2 = *M. levator trochanteris posticus* (D)
- Muscle 3 = *M. remotor trochanteris dorsalis* (D)
- Muscle 4 = *M. depressor trochanteris obliquus* (D)
- Muscle 5 = *M. depressor trochanteris ventralis* (D)
- Muscle 6 = *M. depressor trochanteris medius* (D)

Muscle 7 = M. levator trochanteris anticus (D)

Muscle 8 = M. remotor trochanteris ventralis (D)

I have not confirmed Dillon's M. promotor trochanteris major and minor nor does he show my muscle 1.

NON-MUSCULAR EXTENSION

I agree with Petrunkevitch (1909) and Ellis (1944) that no elevator muscle occur at the femur-patella or tibia-basitarsal joints: as the articulation is almost a dorsal hinge-line such muscles are out of the question. Dillon (1952) agrees that there are no elevators at the femur-patella joint, but suggests that his M. depressor and levator pretarsi (my muscles 28 and 29, fig. 1, D) may act as elevators of the tibia-basitarsal joint. This would only be possible if the articulation were not dorsal, and Dillon in fact figures it as slightly below the dorsal line. I have not confirmed this in the Mygalimorph *Grammostola* spp. (closely related to his *Eurypelma* sp.): here there is a fully dorsal hinge-line as in *Tegenaria*.

It is easy to confirm Ellis's (1944) observations that an increase in hydrostatic pressure in the leg causes elevation at the two joints where elevator muscles are lacking. This can be shown by gently pressing the femur or tibia of a limb either attached to or removed from the body; or by injecting fluid into an animal's body, when all eight legs become fully extended. Ellis suggested tentatively that normal extension might be due to a local rise of pressure the arcuate sclerite (fig. 1, A, B) being raised by a special levator muscle so as to press against the main leg-artery and divert blood into lateral branches in the region of the articular membrane. There are several objections to this idea, at least as applied to *Tegenaria*: (a) it cannot explain extension at the tibia-basitarsus joint, where there is no arcuate sclerite; (b) at the level of the arcuate sclerite in the femur-patella joint the artery is so disposed in relation to the main leg nerve that it is difficult to see how one could be constricted without the other; (c) muscles 19 and 20 (fig. 1, A, B), corresponding to Ellis's levator muscle, are inserted partly on to the sclerite and partly on to the rim of the patella, being favourably placed to produce a flexion force when the joint has already been considerably flexed by the longer muscles inserted on to the sclerite (muscles 14, 15, 16, 17, and 18, fig. 1, A, B).

It is proposed to consider the extensor mechanism of these joints in more detail in a later paper, but two alternatives to Ellis's suggestion may be briefly referred to. Firstly, extension might be due to indirect muscles, as in the insect wing mechanism. In fact extension can be produced by a compression force acting along the hinge line of the joints, but no muscles are suitably placed to produce such forces naturally. Secondly, the flexors might act in antagonism to the blood-pressure of the leg so that extension occurred when the flexors were inhibited. This is consistent with Ellis's observation that dehydrated spiders and those deprived of some blood cannot fully extend their limbs. The rate of extension would be determined by the rate of relaxation of the flexors, subject to a maximum rate determined by the hydro-

dynamics of the system. A possible objection to this idea is that the locomotor demands made upon the circulatory system might not always be consistent with the respiratory demands, though it will be noted that during rapid movement an increase in blood pressure would be expected for *both* respiratory and locomotor reasons. If this explanation proved to be the true one, it would be of evolutionary interest in view of the likelihood that arthropod limbs evolved as turgid appendages (Pryor, 1951), a condition still exhibited in such animals as the Onychophora.

THE AUTOTOMY MECHANISM

'Autotomie' was defined by Frédéricq (1883) as 'mutilation par voie réflexe comme moyen de défense chez les animaux'. Usage has since widened the term to embrace all cases of fracture of limbs and other structures at a specific point where structural adaptations associated with the fracture mechanism and reduction of bleeding are found to occur. It seems undesirable to withhold the term 'autotomy' until a precise reflex has been demonstrated or to employ a complicated nomenclature for other cases, e.g. autospasy, autotilly, autophagy (Wood, F. D. and H. E., 1932).

It is generally accepted that spiders' legs readily fracture between coxa and trochanter, but only when an external force is applied to the distal part of the leg by contact with the ground or web, or by being held in the mouth appendages (Wood, 1926; Bonnet, 1930). It occurs when the animal is trapped by one leg, and also some hours after a leg has been injured. No one has confirmed the presence of the upward-pointing chitinous 'knife' at the proximal end of the trochanter, which, according to Friedrich (1906), cuts through muscle 9 and the coxa-trochanter articular membrane when muscle 10 (his *Beuger des femur*, which he shows running dorso-ventrally) contracts. Wood (1926) examined the legs of arachnids, Opiliones, and Amblypyges and concluded that there was no special adaptation for autotomizing the legs, but that fracture occurred, as a result of external forces, at the weakest joint which was the coxa-trochanteral in arachnids, the trochanter-femural in Opiliones, and the patella-tibial in Amblypyges. She did not explain why the weakest joint was in a relatively thick part of the leg and not nearer the distal end. In scorpions the legs did not easily fracture and there was no specific locus of severance.

Bonnet (1930), working on the pisaurid spider *Dolomedes plantarius*, says that all the coxal muscles except muscle 9 are inserted on to the articular membrane of the coxa-trochanter joint and that when the limb fractures these muscles pull the membrane inward towards the coxa. He also describes a chitinous ring, in two parts, which detaches from the trochanter with the articular membrane and borders the opening.

I have shown above (see fig. 3, A-D) that in *Tegenaria* the coxal muscles are all inserted on to a ring of sclerites which fit into a groove in the proximal rim of the trochanter. The joint fractures between these sclerites and the trochanter, and the coxal muscles then pull the articular membrane proximally

while at the same time the sclerites converge on one another (fig. 3, E) so that a comparatively small hole is left in which the blood rapidly clots and which after a day or two is sealed by a brown plate. The fact that the leg fractures (though the membrane is not withdrawn to the same extent) when the animal is narcotized or freshly killed indicates that active contraction of the coxal muscles is not essential in causing the actual fracture. However, when an external force is applied to the end of the leg the opposing passive resistance of the coxal muscles must set up a strain where the sclerites are attached to the trochanter, and thus cause the fracture to occur there. The function of the oblique sclerite (fig. 3, B, E, f) is not clear: possibly this also causes a local strain at its tip between sclerites *c* and *d*, and thus contributes to the fracturing process.

The autotomy mechanism described by Bonnet and myself differs in principle from that found among insects and Crustacea. Among insects the phasmids are particularly liable to autotomy and here (Bordage, 1905) the fracture occurs at the level of the junction of trochanter and femur which are fixed relative to one another; and a double diaphragm occurs at that point and limits the bleeding. In decapod crustacea (Wood, F. D. and H. E., 1932) a plane of weakness occurs near the proximal end of the ischium (not at the joint) and there is a diaphragm just proximal to this point. In neither insects nor Crustacea do any muscles or tendons cross the plane of fracture, and there is nothing remarkable in this since the fracture does not occur at a functional joint. In spiders only one small muscle (muscle 9) crosses the fracture plane despite the fracture plane being at a *functional* joint. This is achieved by the insertion of the coxal muscles, not on to the trochanter itself but on to the sclerites which separate from the trochanter when fracture occurs, thus enabling the articular membrane to function as a diaphragm which reduces bleeding.

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The Physical Nature of the Lipid Globules in the Living Neurones of *Helix aspersa* as indicated by Measurements of Refractive Index

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With one plate (fig. 1)

SUMMARY

The lipid globules in the neurones of *Helix aspersa* have been found by Chou in 1957 to be of three distinct kinds that differ from each other in chemical composition. In the present investigation, the refractive indices of these three kinds of globule were measured by the technique for measuring the refractive indices of cytoplasmic inclusions in living cells developed by Ross in 1954, which is here described in detail.

It was found that the refractive indices of the globules containing mixed lipids and proteins and those that probably contained triglycerides alone, all had relatively high refractive indices of about 1.47–1.50. These values are comparable with the known refractive indices of many pure lipids.

The refractive indices of the globules containing phospholipid were much lower, being about 1.41–1.42, which is lower than that of any pure lipid. This indicates that these globules probably also contain appreciable amounts of water associated with the phospholipid molecules, as was suggested by Schmidt in 1939.

The results also show that refractive index measurement made in conjunction with histochemical investigations may, in some cases, provide useful additional information about the physico-chemical nature of cell constituents.

INTRODUCTION

It has recently been shown by Chou (1957) that there are three different kinds of lipid globules in the neurones of the snail, *Helix aspersa*, and that they are clearly distinguishable from each other by histochemical tests. The kinds are as follows:

(1) Rather irregularly shaped yellow globules (*y* in fig. 1, A), 1–2 μ in diameter, found throughout the cytoplasm of the cell-body but particularly concentrated in the regions of the axon hillock. These are chemically complex bodies containing mixed lipids, carbohydrate, and protein.

(2) Spherical colourless globules (*c* in fig. 1, A) about 0.8 to 1.4 μ in diameter, found throughout the cytoplasm of the cell-body, but particularly abundant in the proximal part of the axon where the other two types of globule are usually absent. These probably contain only triglycerides, because they react negatively to all histochemical tests except the Sudans, which suggests that other substances are unlikely to be present. There is unfortunately no positive histochemical test for triglycerides.

(3) Nearly spherical 'blue' globules (*b* in fig. 1, A) about 1 to 2 μ in diameter uniformly distributed throughout the cytoplasm of the cell-body. These are actually colourless in the living cell but are distinguishable from the colourless globules described above by the fact that they are stained blue in life by methylene blue, brilliant cresyl blue, and Nile blue. They are found to contain phospholipid.

Under the phase-contrast microscope, the yellow and colourless globules in the living cells immersed in saline presented an appearance typical of rather refractile objects, such as might be expected if they consisted of concentrated proteins and lipids, or pure lipids. The 'blue' globules, on the contrary, seemed strikingly less refractile, and this suggested that the phospholipid they contained might be associated with water.

It was thought desirable to make actual measurements of the refractive indices of each of the kinds of globules to see if this were true.

METHOD AND MEASUREMENTS

The method used for measuring the refractive indices of the globules was the same as that developed by Ross (1954) for measuring the refractive indices of the cytoplasmic inclusions in living cells. An interference microscope was used for measuring the retardation in phase of the light passing through the centre of each globule compared with the light passing through an adjacent region. The diameter of each globule was also measured and its refractive index was derived from these two measurements. It was, however, first necessary to immerse the cell itself in a mounting medium that had the same refractive index as the cytoplasm in order to ensure that the phase-change

FIG. 1 (plate). Living neurones of *Helix aspersa* photographed under a Smith interference microscope, with an Ilford 807 (mercury green) filter.

A, neurones from the ventral ganglion, mounted in 0.7% saline, showing three different kinds of lipid globules. *y*, yellow globules; *c*, colourless globules; *b*, 'blue' globules.

B, a portion of a large neurone from the ventral ganglion mounted in 0.7% saline showing the axon hillock, *n*, and the axon, *a*, containing 'colourless' lipid globules, *c*, arranged in rows. A large mass of the same colourless globules, *m*, is visible near the axon hillock.

C, neurones from the ventral ganglion mounted in an isotonic protein medium with a refractive index of 1.358. In this photograph and in D and E the cytoplasm of cells 1, 2, 3, and 4 appears invisible, indicating that in these cells it has a refractive index equal to that of the mounting medium. In the remaining cells the cytoplasm is to some extent visible. The analyser goniometer of the microscope is set at 150°, giving a maximally bright field.

D, the same group of cells as in C, with the analyser goniometer set at 70°, giving a maximally dark field.

E, the same group of cells as in C and D, with the analyser goniometer set at 120°. At this setting, some of the lipid globules in cells 1, 2, 3, and 4 appear nearly maximally dark. The rotation of the analyser from the position in D indicates a phase-change of 100°.

F, a single neurone from the dorsal ganglion mounted in an isotonic protein medium with a refractive index of 1.361, with the analyser set to give a maximally dark field. The cytoplasm appears almost invisible, but the lipid inclusions show up bright.

G, the same neurone as in F, with the analyser set to give a maximally bright field.

C, D, and E were photographed under a 4-mm 'shearing' objective. A, B, F, and G were photographed under a 2-mm 'double focus' objective. One small division of the eyepiece scale in C, D, and E = 3.7 μ . One small division of the eyepiece scale in F and G = 1.7 μ .

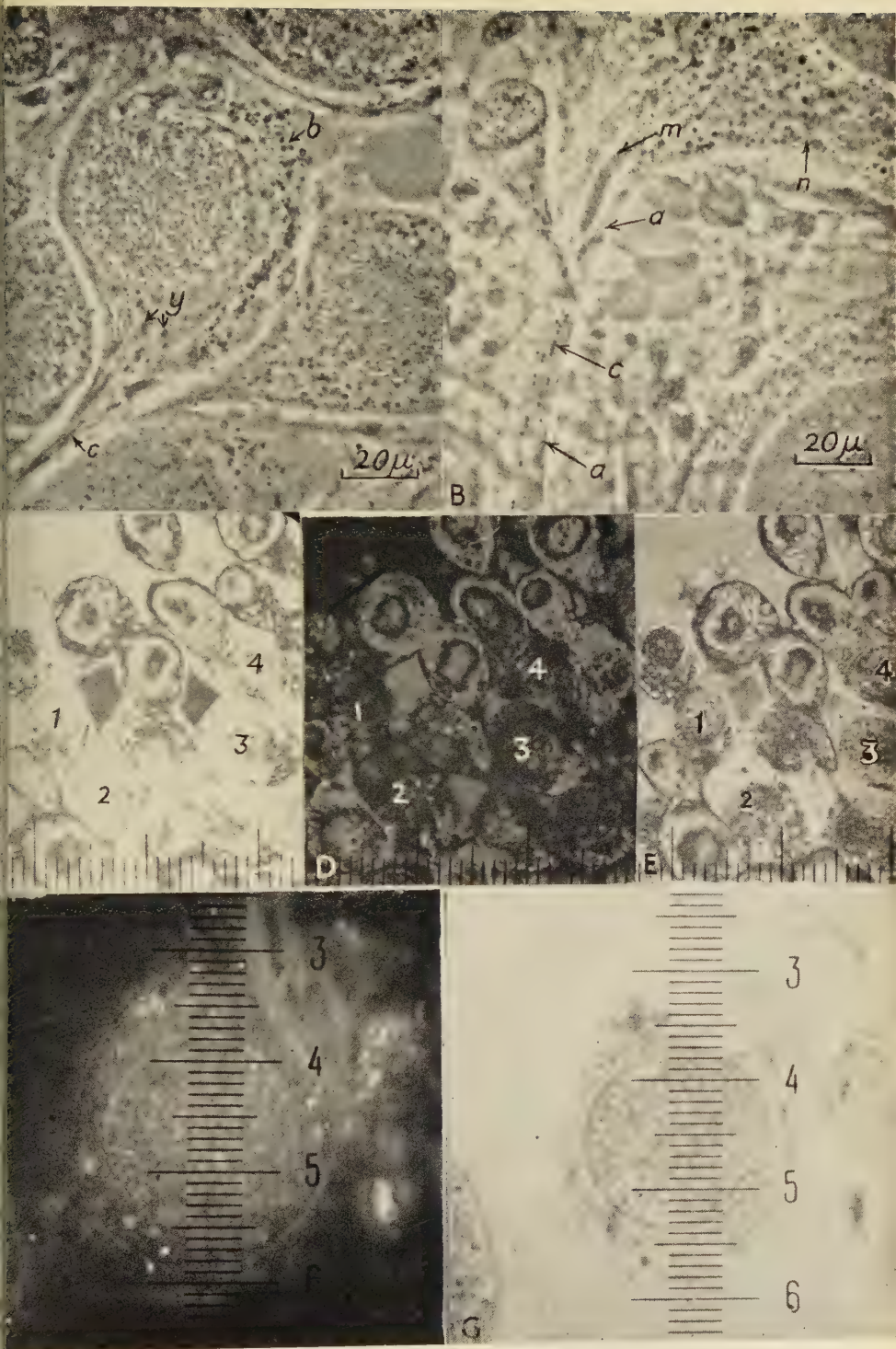


FIG. 1

K. F. A. ROSS and J. T. Y. CHOU

measurements made on the globules were unaffected by phase changes in the surrounding cytoplasm.

Suitable mounting media were made from a 20% solution of Armour's bovine plasma albumin, fraction V in 0.5% saline, which has a tonicity approximately equal to that of a 0.7% saline solution, which is isotonic with snails' blood (Ross, 1952). A series of dilutions of this protein solution were then made by adding 0.7% saline, and their refractive indices were measured with a Bellingham and Stanley pocket refractometer. This instrument has a built-in filter giving maximum transmission at $589\text{ m}\mu$. It is sufficiently accurate to give figures of refractive index that are reliable to the third place of decimals. This third place is unlikely to have been affected by variation in room-temperature during the course of the observations recorded in this paper.

Living neurones were obtained from the cerebral ganglion and ventral ganglion-mass, removed from the freshly decapitated snail. Pieces of this tissue were teased in each of the protein solutions and the cell suspensions were examined under a Smith interference microscope (manufactured by Messrs. Charles Baker of Holborn). It was found that in the protein solutions with refractive indices between 1.357 and 1.364, some neurones could be seen in which the cytoplasm appeared to be of exactly the same interference colour as the background, while, if nearly monochromatic light were used, the cytoplasm matched the background intensity at all settings of the analyser (fig. 1, C, D, and E). This meant that in these neurones, the cytoplasm had exactly the same refractive index as the mounting medium (or had a refractive index within 0.001 of this value). Phase-change measurements could therefore be made on the inclusions in those cells without it being necessary to take into account the refractive index and thickness of the surrounding cytoplasm.

Phase-change measurements (in the direction of the optical axis of the microscope) were made with the interference microscope by the extinction point method in green light with a measured mean wavelength of $542\text{ m}\mu$, which was obtained by using a tungsten 'pointolite' lamp and an Ilford 807 (mercury green) gelatine filter. The analyser of the microscope was first turned until the background and cytoplasm appeared maximally dark (fig. 1, D) and then turned again until the centre of the globule appeared maximally dark (fig. 1, E). With this material, phase-change measurements could thus be made accurately to the nearest 6° (a sixtieth of a wavelength).

The diameter of the globules (in the direction at right angles to the optical axis of the microscope) was estimated to the nearest $0.2\text{ }\mu$ by means of an eyepiece micrometer scale. With the 2 mm ('double-focus') objective used, however, such measurements are only reliable to about the nearest $0.4\text{ }\mu$, and this was the limiting factor in determining the accuracy of the refractive index measurements. A rather more accurate estimate was possible in the case of the colourless globules because those in the axon were often found to be arranged in straight rows of up to a dozen or so globules, apparently of identical size, so close together that they appeared to touch each other (fig. 1, B). In these cases, the maximum diameter of an individual globule can be accurately determined

by measuring the overall length of a row (to the same degree of accuracy as is possible to measure an individual globule) and dividing by the number of globules. It was, of course, possible that the globules did not quite touch each other and were therefore rather less in diameter than they appeared, but the upper size-limit could be clearly defined.

The refractive index (n) of each globule was calculated from the formula

$$n = \frac{\phi}{360} \times \frac{\lambda}{d} + m,$$

where ϕ = the measured retardation in phase of the light passing through the centre of the globule (expressed as an angle), d = the diameter of the globule estimated by eyepiece micrometer to the nearest 0.2μ , λ = the mean wavelength of the light used (0.542μ), and m = the refractive index of the protein mounting medium (and of the cytoplasm of the neurone in which the globules were measured). Since the two sets of measurements from which the refractive indices were derived were made in directions at right angles to each other, it was assumed that the globules were perfectly spherical and this was nearly true even in the case of somewhat irregularly shaped yellow globules.

Before they were measured, the globules were individually identified: white light with the polarizer of the interference microscope in the 'out' position, and the microscope stage was moved until they were in an easily recognizable position in relation to the eyepiece micrometer scale for subsequent measurements. The yellow globules and the colourless globules in the axon were quite easy to recognize, but for the certain identification of the 'blue' globules it was necessary to use cells which had been supravital stained with Nile blue before being mounted in the protein solution. With the green filter used, it was extremely unlikely that the blue-green coloration of these globules affected the phase-change measurements because they were invisible when viewed with a green filter and no polarizer.

A total of 10 yellow globules were measured in two different cells with matched cytoplasm mounted in a protein medium with a refractive index of 1.358; and 5 colourless globules were measured in the axon region in a single matched cell in a similar mounting medium. Five 'blue' globules were measured in two other matched cells in another preparation where the refractive index of the mounting medium was 1.361.

Table 1 shows the measurements of the phase-change and diameter made on each globule and the refractive indices calculated from these. The final column of the table shows the upper and lower limits of the refractive index of each globule, on the assumption of an error of $\pm 0.2 \mu$ in the diameter measurements. It is very unlikely that the refractive indices of any of the globules lay outside these limits, and indeed, these extreme errors themselves are most unlikely. It is probable that the refractive indices of the colourless globules were not as low as the lower limit shown because those that were measured were all either arranged in rows in the manner described above or

were indistinguishable in diameter from those that were in rows. These values are therefore placed in brackets.

TABLE I

Measurements of the phase changes and diameters of lipid inclusions in the neurones of *Helix aspersa*, and their refractive indices calculated from these values

Measured phase retardation through globule (degrees)	Diameter of globule to the nearest 0.2 μ (measured by eye-piece micrometer)	Refractive index of globule from ϕ and d	Range of refractive index of globule, assuming a maximum error in diameter measurement
ϕ	d	n	(t) of $\pm 0.2 \mu$
a) Yellow globules in the cell-body in a matching medium of r.i 1.358			
160	1.8 μ	1.490	1.479-1.510
120	1.6 μ	1.471	1.460-1.489
100	1.2 μ	1.484	1.466-1.510
76	1.2 μ	1.454	1.439-1.474
100	1.2 μ	1.484	1.466-1.510
128	1.4 μ	1.496	1.475-1.519
118	1.6 μ	1.470	1.457-1.486
90	1.2 μ	1.471	1.455-1.495
100	1.4 μ	1.466	1.453-1.485
96	1.0 μ	1.504	1.479-1.541
Mean refractive index of yellow globules = 1.479			
b) Colourless globules in the axon in a matching medium of r.i 1.3585			
110	1.2 μ	1.496	(1.477)-1.526
100	1.2 μ	1.484	(1.466)-1.510
84	1.0 μ	1.485	(1.464)-1.517
98	1.0 μ	1.506	(1.480)-1.544
82	1.0 μ	1.482	(1.461)-1.514
Mean refractive index of colourless globules = 1.491			
c) 'Blue' globules in the cell body in a matching medium of r.i 1.361			
50	1.4 μ	1.415	1.408-1.424
40	1.2 μ	1.411	1.405-1.424
34	1.0 μ	1.413	1.404-1.426
44	1.2 μ	1.416	1.409-1.428
66	1.6 μ	1.423	1.416-1.433
Mean refractive index of 'blue' globules = 1.416			

CONCLUSIONS

From table 1 it will be seen that the colourless globules have a mean refractive index of 1.491. This value is closely comparable with the refractive indices, at room temperature, of many fats and oils composed mainly of triglycerides, e.g. cotton seed oil (1.475), or olive oil (1.466). Indeed, at room temperature, nearly all fats and oils of vegetable or animal origin have refractive indices between 1.46 and 1.48 (Hodgman, 1945), so that the values are, if anything, rather higher than one might expect.

The refractive indices of the yellow globules are rather more variable, as

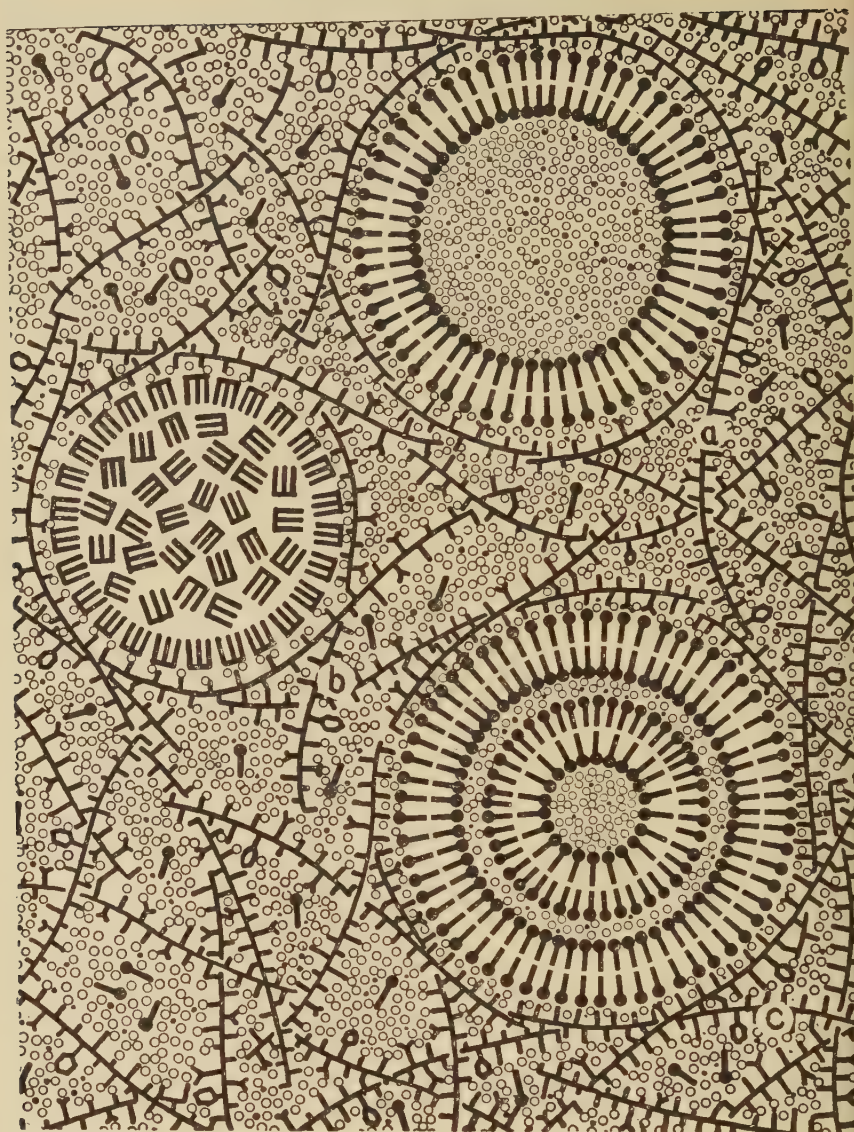


FIG. 2. An interpretation of the submicroscopic structure of protoplasm. Y protein; \bullet phospholipids and related substances; W triglycerides; \circ water molecules; \cdot ions. *a*, a vacuole with aqueous contents surrounded by a bimolecular phospholipid lamella; *b*, a triglyceride droplet; *c*, a phospholipid droplet; between these droplets is a protein framework which holds in its meshes water and other substances. From Schmidt (1939).

might be expected in view of their rather complex chemical composition, but these values also are those that might reasonably be expected if these globules consist mainly of lipid material or rather concentrated protein. Their mean

refractive index was 1.479, which was only a very little lower than that of the colourless globules.

The 'blue' globules on the contrary had very much lower refractive indices with a range which, even allowing for the maximum error in measurement, does not overlap those of the other two kinds of globule. Their mean refractive index was 1.416. This is lower than that of any pure lipid including phospholipids, although histochemical tests show the presence of no substance other than phospholipids. The latter, however, differ in one important respect from triglycerides in that they have a side chain in their molecule (the phosphoric acid / choline radicle) that is hydrophil. This means that, unlike triglycerides, they will associate with water molecules, and the low refractive index measurements suggest that in addition to phospholipid, the 'blue' globules probably contain quite appreciable amounts of water in an association of this kind.

Schmidt in 1939 used polarized light to investigate living cell structure and concluded that phospholipid and similar molecules tended to form bimolecular layers with the hydrophil chain orientated towards an aqueous phase (*a* in fig. 2). In spherical droplets containing phospholipid, he found a distinct 'polarization cross' which was absent in similar droplets composed of triglyceride. This indicated that while the triglyceride molecules were probably disorientated except at the surface of the droplet (*b* in fig. 2), the phospholipid molecules appeared to be radially orientated throughout the droplet from its surface to centre (*c* in fig. 2). This led him to suggest that such droplets probably had a series of concentric shells of water in between bimolecular layers of phospholipid molecules (*c* in fig. 2).

Our present results are in agreement with Schmidt's hypothesis in that they strongly suggest that the globules containing phospholipid in the neurones of *H. aspersa* must also contain a considerable amount of water. They also indicate that there may be other useful possibilities in applying these refractometric techniques in conjunction with histochemical investigations for determining the physico-chemical nature of cell constituents.

We are very much indebted to Dr. J. R. Baker for suggesting this investigation, which was carried out in his laboratory at Oxford, and for his invaluable advice and encouragement. The interference microscope for the work was provided out of a grant to one of us (K. F. A. R.) from the London University Central Research Fund; J. T. Y. C. was on an Inter-University Council Fellowship through the Carnegie Corporation of New York and on leave from the Department of Zoology, University of Hong Kong.

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The Formation and Properties of the Organic Matrix of Reptilian Tooth Enamel

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With two plates (figs. 1 and 2)

SUMMARY

A number of features of enamel formation in the lizard *Agama atricollis* are described. The behaviour and properties of the ameloblasts indicate that the process of enamel formation is similar to the corresponding process in mammals; the fibrous enamel matrix appears to be formed from outgrowths of the cytoplasm of these cells. Interprismatic material, as it is known in mammals, is not produced, so that reptilian matrix tends to be uniformly fibrous. Nevertheless, the fibres are initially arranged in groups corresponding to the ameloblasts. There is no distinct pre-enamel stage because matrix production is immediately followed by a limited influx of mineral in an elementary state, converting the matrix into an basiphil form. Striae of Retzius may be due to periodic pauses in the normal process of matrix production enabling the ameloblasts to assimilate and secrete mineral. Before the onset of final calcification, the matrix seems to undergo a modification rendering it capable of influencing the size and orientation of mineral crystallites.

The organic matrix has a refractive index of 1.57 and has no intrinsic birefringence. However, in suitable liquids the parallel fibres produce a positive form birefringence. If paraffin wax is allowed to crystallize on the matrix, optically negative streaks are formed parallel with the fibres, perhaps as the result of crystal overgrowth.

Evidence obtained indicates that this reptilian type of ectodermal enamel is a likely precursor of the mammalian prismatic type. The evolution from one to the other could have been achieved in a comparatively simple step.

INTRODUCTION

THE nature of reptilian enamel is of interest in that it has now been established that two quite different types of enamel may be found covering the teeth of different vertebrates. Mammalian enamel is prismatic in structure and is produced by the ectodermal cells of the enamel organ, whereas the enamel covering the teeth and scales of fish (Kvam, 1946, 1950; Levi, 1939, 1940a; Kerr, 1955; Poole, 1956a) and that covering the teeth of amphibians (Kvam, 1946; Levi, 1940b) is not prismatic and its matrix is formed by the mesodermal odontoblast cells of the dentine papilla. Although the teeth of a wide range of reptiles have been examined (Erler, 1935; Schmidt, 1947; Poole, 1956b), as yet no prisms have been found in the enamel; however, there is some evidence that the enamel matrix of reptiles has certain staining properties in common with mammalian matrix (Kvam, 1946).

Very many accounts of mammalian enamel formation are to be found in dental and zoological literature; summaries of the most important details are

given by Kvam (1946) and Marsland (1951, 1952). It may be briefly stated here that two main stages are recognizable in enamel formation—the production of an organic matrix with only a slight mineral content, followed by a maturation process by means of which this matrix becomes highly calcified.

The organic matrix is formed by the ameloblasts of the enamel organ. The so-called Tomes's processes seem to be intimately connected with the formation of the matrix. The two most popular views are that either these processes secrete the matrix, or that they are converted into the matrix as they grow out from the basal ends of the ameloblasts. During its development, the properties of the matrix change a number of times. Soon after deposition it is acidophilic and is known as pre-enamel; an influx of calcium salts, probably organic colloidal in form, produces a change to intense basophil properties. The enamel matrix remains in this condition, with a mineral content not greater than 35% (Weinmann, Wessinger, and Reed, 1942), until it has reached the final thickness of the future enamel. Immediately before the final heavy influx of mineral a return to acidophil properties is shown, but, as calcification proceeds, there is a withdrawal of organic material and water, the residual matrix becoming soluble in acids. As enamel production occurs, certain changes also take place in the ameloblasts including alterations in the size, shape, and contents of the cells, and in the position of the nucleus (Marsland, 1951).

The finished enamel shows incremental lines, the striae of Retzius, which may also be seen in the matrix before its final, heavy calcification. These appear to be related in some way to matrix production (Marsland, 1951) and the direction of calcification is almost at right angles to these lines. Alkaline phosphatase distribution follows a regular pattern in developing mammalian tooth-germs, although different authors have reached slightly different conclusions about distribution in ameloblasts and odontoblasts. A survey of these results is given by Symons (1955) who concludes that alkaline phosphatase may be initially concerned with cell differentiation and growth rather than with calcification.

In a fully calcified reptilian tooth the enamel is made up of incremental layers which follow approximately the shape of the tooth crown. As in mammals, this pattern is due to the presence of striae of Retzius. Mineral crystallites tend to be arranged at right angles to the layers, and whilst there is an irregular variation of crystallite direction in crocodile enamel (Erler, 1935), an extremely regular variation occurs in the enamel of placodonts (Schmidt, 1947*b*), gorgonopsids, and cynodonts (Poole, 1956). A regular variation produces a prismatic appearance under certain conditions of examination, but no true prisms are found.

Fibres have been described in sections of fully formed crocodile enamel (Kvam, 1946) which are said to cross one another in three planes (Marcus, 1931; Schulte, 1930). Erler (1935) was unable to confirm a three-dimensional fibre network, but found air-filled spaces in the enamel impermeable to liquids. Such air spaces occur in the enamel of many reptiles, fossil and recent (Schmidt, 1947*a*; Poole, 1956*b*). It is possible that some of these structures

features have been confused by different authors; Kvam (1946), for example, states, 'longitudinally to the tooth there are undulating fibres'; but neither in the text nor in the illustrations does he distinguish between these and the striae of Retzius.

During development the organic matrix of the enamel of lizards and mammals stains red with Heidenhain's Azan, contrasting with mesodermal enamel of lower vertebrates, which stains blue in the same way as the collagenous dentine (Kvam, 1946). Because of this it is suggested that reptilian enamel has a keratinous nature rather than collagenous and, therefore, is ectodermal in origin rather than mesodermal.

The object of the work to be described here has been to make a more detailed examination of the formation and properties of reptilian enamel matrix, and to see how far such properties compare with the enamel matrix of mammals.

MATERIAL AND METHODS

The results given here were obtained from the examination of embryonic jaws of the lizard *Agama atricollis*. This lizard was chosen because of the relative ease with which eggs are obtained at certain times of the year, but some other lizards including the local gecko (*Hemidactylus* sp.) and skink (*Mabuia varia*) were also studied briefly, as well as a few embryos of the crocodile (*Crocodilus niloticus*). In all these cases the properties of the enamel matrix were the same. As there appears to be no complete account of the structure of a fully formed lizard-tooth, sections of the teeth of the local monitor lizard (*Varanus niloticus*) were prepared by grinding and examined under the polarizing microscope. The structure of the enamel was so similar to crocodile enamel that a description is not necessary, but the range of reptiles possessing a similar enamel structure (Poole, 1956b) must now include modern Lacertilia.

One or more agamid eggs from a clutch were opened at weekly intervals and the embryos fixed in either Bouin or Susa. Unfortunately, the series of embryos produced was not regular since the rate of development varied from egg to egg. Nevertheless, enough stages were found to provide the main landmarks in tooth formation. After fixation, no further decalcification was required.

Under the prevailing local climatic conditions, difficulties were experienced with the normal technique of dehydrating and clearing, particularly with bulk tissue about to be embedded. To overcome this, dehydration was carried out with mixtures of ethyl and *n*-butyl alcohol. The tissue to be embedded was transferred from absolute butyl alcohol into a mixture of butyl alcohol and paraffin wax (m.p. 54° C) and finally into pure paraffin. The technique proved to be very successful, since no appreciable hardening occurred even when the material was left to embed overnight. Sections on the slide were cleared by passing them from 95% alcohol through terpineol into benzene.

Standard sections of each embryo were prepared and stained with

haematoxylin and eosin. Harris's haematoxylin was preferred to others since it gave more delicate differentiation of tissues than Heidenhain's, and yet was more stable than Delafield's. Some sections were also stained with Azan, which, as previously mentioned, is said to give a colour differentiation between keratin and collagen.

Finally, alkaline phosphatase tests were made by the technique of Gomori (1939) as modified by Danielli (1946). Again the dehydration and embedding technique was modified by using butyl alcohol. To reduce the time required for embedding, a vacuum oven was used and the pressure lowered with a water pump. An embedding time of $1\frac{1}{2}$ h gave satisfactory results.

GENERAL FEATURES OF THE TEETH AND TOOTH-GERMS

Agamid lizards have an acrodont dentition, each tooth being ankylosed to the upper edge of the jaw. The tooth tips are flattened laterally and triangular in shape so that each jaw has the appearance of a continuously serrated cutting edge. The embryonic jaws of *Agama atricollis* possess nine teeth at hatching compared with about twice this number in the adult jaw. During the post-hatching growth period new teeth are produced one by one in a pocket at the back of the jaw and, as the jaw increases in length, new teeth are added at the end of the row. This is also found in acrodont chameleons (Rose, 1892). No evidence has arisen from the study of *Agama* to contradict the general belief that teeth are not replaced in acrodont forms. Nevertheless, although the teeth are all similar in size at hatching, the second tooth in each adult jaw is considerably enlarged. Presumably, in this particular case, post-hatching growth is maintained for some time.

The first signs of tooth formation were found in an embryo 28 days old. Here the oral epithelium had invaginated along the length of the jaw and soon afterwards this lamina gives rise to the nine germs of the teeth which will be present at hatching. The appearance of an early tooth-germ is shown in figs. 1, A, B. At this stage it is possible to distinguish an enamel organ consisting of an inner and outer enamel epithelium separated by stellate cells, and a small

FIG. 1 (plate). A series of developing tooth germs of *Agama atricollis* as seen in vertical sections.

A and B, very young germ stained with Heidenhain's Azan. The enamel organ consists of inner and outer enamel epithelia separated by stellate cells. Nuclei are situated centrally in the ameloblasts; between these cells and the odontoblasts is a layer of pre-dentine.

C, enlarging germ stained with haematoxylin and eosin. Enamel formation extending downwards as the bone of the jaw differentiates below.

D, stage similar to C, stained with Azan. Ameloblasts are elongated with nuclei at their outer ends; enamel matrix shows vertical refractive stripes caused by the bunching of fibres.

E, later germ stained with haematoxylin and eosin. Considerable enlargement has occurred and the base of the tooth-germ is approaching the bony platform to which it will eventually become ankylosed. A blood-vessel passes through the bone into the pulp.

F, enlarged base of germ shown in E, where enamel formation is in progress. The inner ends of the ameloblasts show Tomes's processes, which stain in the same way as the cytoplasm of the ameloblasts and project through the terminal cell membrane.

G, enlarged tip of same in which the enamel has here reached its final thickness. No Tomes's processes are present, the inner border of the ameloblasts being smooth.

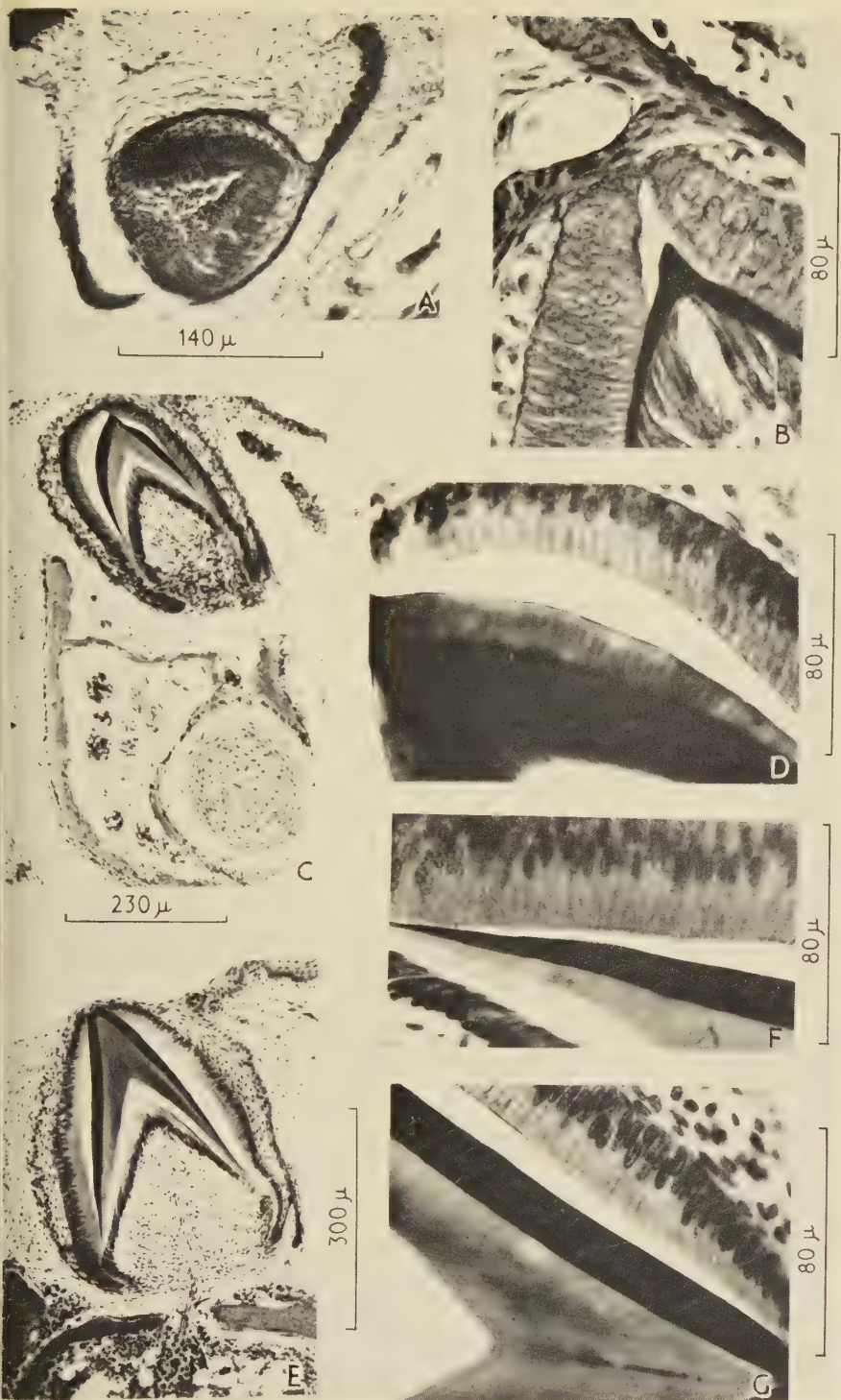


FIG. 1
D. F. G. POOLE

number of odontoblasts differentiated from pulp cells. The organization of the enamel organ is rather simpler than that of crocodile, which, according to Rose (1893), is the only reptile in which the condition of the enamel organ approaches that of mammals. Although the structure of this lizard enamel organ is clearly seen over the tip of young germs, only the inner enamel epithelium is clearly defined at later stages.

The tissue separating the inner enamel epithelium from the odontoblasts in fig. 1, B is pre-dentine. It gives a faint pink colour with haematoxylin and eosin, whilst its collagenous nature is shown by the fact that it stains blue with Azan. Thus, the first tissue produced is dentine and in this respect the reptilian tooth-germ resembles that of mammals and differs from fish, where the mesodermal enamel matrix is completed before dentine formation begins.

The tooth-germ increases in size by the downward extension of the enamel organ; new ameloblasts are continually produced at the base where inner and outer enamel epithelia can be distinguished until later stages. Inside the ameloblasts new odontoblasts appear and lay down pre-dentine. Enamel is laid down over the pre-dentine at the tip and eventually, as the downward extension of the enamel organ occurs (fig. 1, C, E), it is also laid down over the pre-dentine at the sides of the tooth. As the tooth-germ enlarges, a bony platform develops below to which the completed tooth will eventually become ankylosed. Occasionally the bony platform is not continuous (fig. 1, E) and blood-vessels pass from the mesodermal tissue surrounded by the bone, through a gap and into the pulp cavity. One large blood-vessel is always seen to enter the pulp cavity, but the origin of the vessel varies from germ to germ.

Finally, some weeks before hatching, a pocket is formed at the back of the jaw. Into this extends the dental lamina and a new tooth-germ begins to form. This is the first of the teeth which will be added to the series after hatching. Ankylosis of the nine teeth in each jaw also occurs after hatching.

HISTOLOGY OF THE AMELOBLASTS AND ENAMEL MATRIX

Fig. 1, B is an enlargement of the tip of a young germ; a thin layer of pre-dentine is present but, as yet, no enamel has been formed. The ameloblasts are elongated with centrally placed, granular nuclei; the latter are typically basophil and the cytoplasm acidophil. The odontoblasts producing the pre-dentine are also long, but in this case the nuclei are at the inner ends of the cells adjacent to the pulp.

As soon as the enamel appears it has very precise staining properties. The appearance of a germ where enamel formation is under way is illustrated in fig. 1, C. The staining properties of the ameloblasts are unchanged but they are considerably longer and their nuclei are at the outer ends of the cells (fig. 1, D). At the base of the germ no enamel is present and the ameloblasts, with centrally placed nuclei, are shorter than those at the tip. A thin layer of pre-dentine is present beneath them so that the condition at the base of this germ is identical with that over the tip of the young germ described above. Enamel formation is thus preceded by the elongation of ameloblasts, the

migration of the nuclei to the outer part of the cell, and the deposition of a layer of pre-dentine. These changes proceed gradually as enamel formation extends downwards from the tip of a germ.

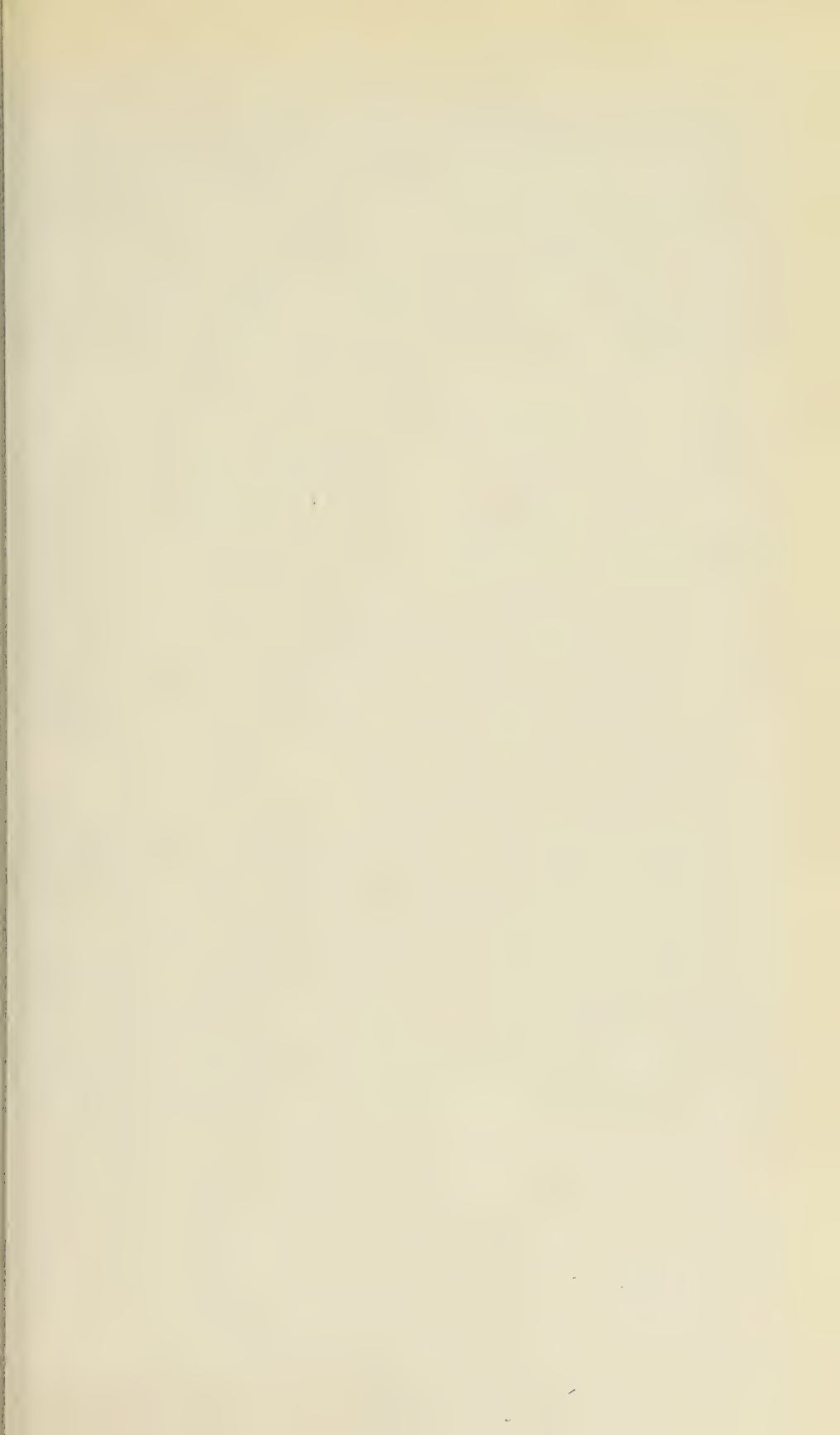
The enamel matrix which is present at this stage stains so intensely with haematoxylin that little or no structure may be seen in it. With Azan, enamel becomes a deep red, the same colour as the keratinous scales which develop on the surface of the embryo; this contrasts sharply with the bright blue of dentine and bone. These properties do indeed suggest that the matrix is more like keratin than collagen, as was suggested by Kvam (1946).

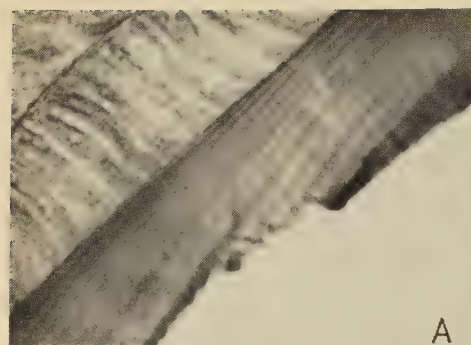
In sections which have been stained with Azan or lightly with haematoxylin the matrix is not of a uniform appearance (fig. 1, D). Fibres are seen throughout and there are alternating light and dark stripes running vertically to the tooth surface and parallel with the general fibre direction. The striped effect results from refraction, for it is seen even in unstained preparations and, moreover, the light and dark areas change when the microscope tube is racked up and down. Opposite each ameloblast a slight bunching of fibres occurs and the refractive index along the axis of a group of fibres is probably slightly different from that between groups, where the density of fibres is somewhat less. Thus, alternating zones of slightly different refractive index are found which produce the refractive stripes. Although the cytoplasm is acidophil, the ameloblasts present rather a similar pattern of darker intracellular contents separated by lighter intercellular zones, as may be seen in fig. 1, D. Fibres running in directions other than that described above have not been observed.

The appearance of the inner ends of the ameloblasts is also important. Because of distortion during the various treatments, the ameloblasts are frequently pulled away from the enamel, and in such places the inner surface of this cell-layer is seen to be very irregular (figs. 1, F; 2, B, D). This irregularity is due to the fact that the end of each ameloblast projects through a terminal membrane into the space caused by the distortion. However, where the ameloblasts and enamel are closer together it is possible to see that these projections pass across and are continuous with the fibrous matrix, and it is evident that rupture of the connexion is only produced by severe distortion (fig. 2, B). Similar cell projections are well known in mammalian enamel formation as Tomes's processes.

If an unstained section is examined with phase contrast, the fibrous properties of the matrix are again apparent and the continuity of the ameloblasts with the matrix in an undistorted area is especially clear. From the base of each ameloblast a zone of the matrix runs out towards the amelodentinal junction, gradually fanning out and becoming rather more fibrous as it extends deeper into the zone of enamel.

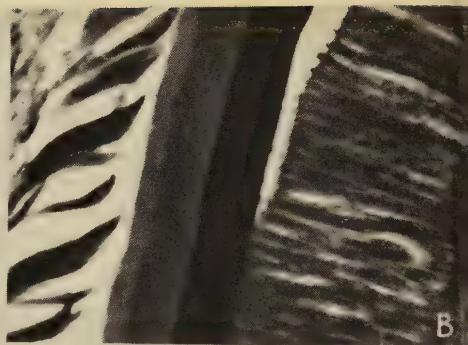
It is important to note here that in areas where the enamel and ameloblasts have not been pulled apart, Tomes's processes are not visible and the deeply staining, basiphil matrix extends right up to the bases of the ameloblasts. Where Tomes's processes do occur, they stain in exactly the same way as the cytoplasm of the ameloblasts (figs. 1, F; 2, B, D). Therefore it seems very





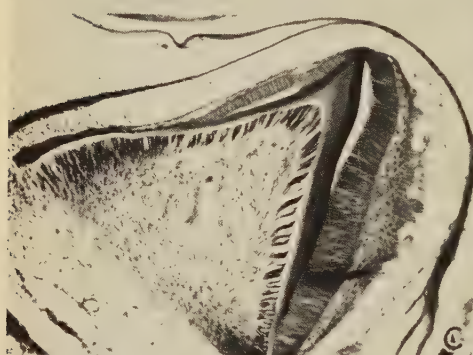
A

60 μ



B

80 μ



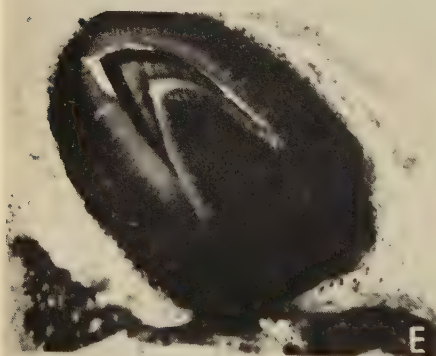
C

300 μ



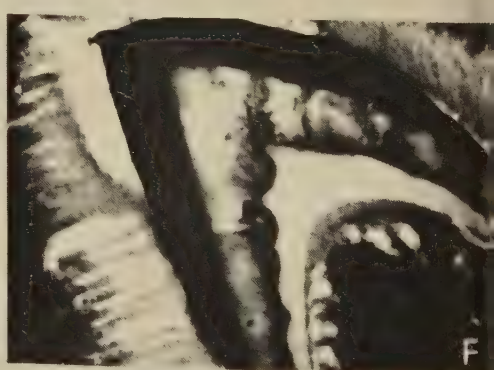
D

80 μ



E

200 μ



F

80 μ

FIG. 2
D. F. G. POOLE

likely that Tomes's processes are artifacts caused by distortion, and really represent the basal parts of the ameloblast cytoplasm which have been pulled out beyond the margins of the cells. However, Tomes's processes are only apparent during the production of the matrix; in fig. 1, C, E, they are present over the developing enamel along the sides of the tooth, but are absent over the tip where the matrix has reached its final width. This is illustrated in fig. 1, F, G. Thus, when matrix production has ceased, there is no longer continuity between the contents of the ameloblasts and the enamel matrix, and Tomes's processes are no longer produced.

Fig. 1, E is of an advanced stage of tooth formation. The staining properties of the matrix remain unchanged but it is now uniformly fibrous in appearance, the bunching having disappeared. This has probably arisen from the untwisting of the fibres. Although they cannot be seen in the illustration, the matrix now shows incremental zones which are evident in the fully formed enamel of all reptiles. Wide zones with the typical staining properties of the matrix are separated by lines which do not stain so readily. These lines also have a slightly different refractive index from the rest of the matrix, for even in unstained sections they can be made to appear light or dark by moving the focus of the microscope. The matrix fibres pass uninterruptedly across the striae and, as shown in fig. 2, A, these properties are seen most clearly in the matrix immediately before the final calcification. Fig. 2, A illustrates another important feature for, at this stage, there is a very rigid attachment between the ameloblasts and enamel matrix, with the result that distortion now tears the matrix away from the dentine. Therefore, although continuity between the matrix and the contents of the ameloblasts disappears at the end of matrix formation, there is a later re-attachment of the ameloblastic terminal membrane to the surface of the matrix. Similar features have been described in the formation of mammalian enamel (Marsland, 1951).

At the hatching stage, no matrix can be seen over the tip of the tooth, although remains of the matrix are found along the sides. Fibres and incremental layer lines may still be recognized in these remains, whose staining properties are somewhat less basiphil than in younger stages. It is suggested

FIG. 2 (plate). Vertical sections of developing tooth-germs of *Agama atricollis*.

A, ameloblasts and enamel shortly before the onset of final calcification, stained with Azan. The enamel matrix, which is now uniformly fibrous and shows striae of Retzius, is so firmly attached to the ameloblasts that distortion causes a fracture at the amelodentinal junction.

B, part of a germ where matrix formation is still in progress, stained with haematoxylin and eosin. The shape of Tomes's processes is seen to depend upon the degree of distortion of the germ; a number are still connected to both ameloblasts and matrix, but where distortion is absent the basiphil matrix extends right up to the bases of the ameloblasts.

C and D, side of a germ over the mid-line of which enamel formation has ceased and the residual matrix has become acid-soluble. Here the tooth is triangular in section; enamel and dentine formation are still in progress, the ameloblasts showing clearly a basal membrane and Tomes's processes. Processes from the odontoblasts pass into the light coloured pre-dentine. Stained with haematoxylin and eosin.

E and F, alkaline phosphatase distribution. Most of the germ shows intense activity but neither the ameloblasts nor odontoblasts stain throughout. Pre-dentine is unstained, enamel is black and calcified dentine, the inner border of which is globular in outline, appears grey.

that, as calcification proceeds, a large part of the organic matrix is resorbed, but it is unlikely that resorption is complete and, in fact, a slight organic residue can be obtained by extremely slow and careful decalcification of crocodile enamel. This residual organic material becomes soluble in all but the most dilute acids. Finally, the ameloblasts have again altered in shape, being now much shortened with each nucleus appearing to occupy most of the cell. However, at the side of such a tooth as this, development is at an earlier stage and shows all the features of a young germ (fig. 2, C, D); lateral expansion thus continues until the base of a tooth reaches its final size.

From the above description it is evident that developing reptile enamel has a number of features in common with that of mammals. The staining reaction with Azan is similar in the two cases and there is little doubt that the reptilian matrix, like mammalian, is produced by the ameloblasts. The Tomes's processes of the reptilian ameloblasts are suspected to be artifacts; a similar suggestion has been made in the case of mammals (Kvam, 1946, and others), where the size and shape of the processes is also related to the degree of distortion of the germ. However, the evidence for this is not quite conclusive, with the result that the Tomes's processes are still regarded by some as a natural feature of the ameloblasts, intimately related to matrix production (Marsland, 1951). In *Agama* the staining properties of these processes are the same as those of the cytoplasm of the ameloblasts and, therefore, it seems probable that as distortion takes place, the cell contents are pulled out beyond the cell margins. Yet the inner border of the ameloblasts is marked by a distinct membrane which remains in position even after distortion, so that, if Tomes's processes are produced as suggested here, a means must exist for the cytoplasm of the ameloblasts to pass through the membrane without rupturing it. The terminal membrane in mammals is perforated, spaces alternating with condensations of intercellular material known as the terminal bar apparatus. A similar feature has not yet been recognized in *Agama*, although the existence of small perforations of some sort might be postulated to account for the production of Tomes's processes.

Although they differ in staining properties, there is no distinct structural demarcation between the reptilian matrix and Tomes's processes; the fibres are less distinct in the outermost matrix layers, and gradually merge into the granular processes. The fact that only severe distortion will cause complete rupture illustrates the intimacy of the connexion between fibres and processes. For these reasons it is suggested that the matrix is produced by the basal regions of the ameloblasts which tend to grow out through the terminal membrane and gradually become converted into fibres. Mammalian matrix may also be produced in the same way (Kvam, 1946; Marsland, 1951). In reptiles this process results in an initial bunching of fibres opposite each ameloblast, but eventually the fibres become more uniformly distributed.

Reptilian matrix shows no pre-enamel stage and only a doubtful 'transitional' phase, both of which possess acidophil properties in mammals. The layer tentatively described as pre-enamel in the lizard *Lacerta vivipara*

(Kvam, 1946) could be the same as the zone of Tomes's processes described here in *Agama*, although, perhaps, there is no reason for making such a distinction. Even in mammals there appears to be no clear-cut distinction between pre-enamel and Tomes's processes, except that the latter exist as separated units, and it is also possible for the pre-enamel stage to be missing, as in the mouse (Kvam, 1946). In an attempt to account for these varying details, the following developmental plan is proposed.

In both reptiles and mammals, enamel formation begins with the acidophil contents of the ameloblasts growing out through the terminal membrane and

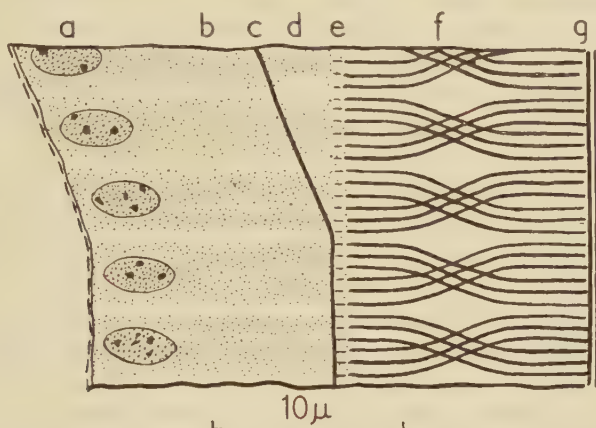


FIG. 3. Diagram of the structure of the matrix and arrangement of the ameloblasts during the formation of reptilian enamel. *a*, granular nuclei of ameloblasts; *b*, cytoplasmic region; *c*, terminal cell membrane, possibly perforated; *d*, Tomes's processes formed in regions of distortion and passing through terminal membrane; *e*, zone where outgrowing contents of ameloblasts are converted into matrix; *f*, fibres of the matrix arranged, initially, in groups corresponding to the ameloblasts; *g*, amelodentinal junction.

becoming transformed into the matrix protein. In reptiles and some mammals, this transformation is rapid and is quickly followed by an influx of mineral converting the matrix into a basiphil form. If, however, the transformation and mineral deposition are delayed, the matrix retains the form and acidophil properties of the cytoplasm and is known as pre-enamel. In either case the matrix remains continuous with the cell contents, so that distortion pulls the cell-walls and terminal membrane away from the matrix, leaving the extenuated cell contents behind as Tomes's processes. The existence of pre-enamel will then depend upon the rate of transformation and initial calcification of the matrix, and the length and shape of Tomes's processes upon the extent to which the ameloblasts are pulled away from the matrix. Thus, until conversion occurs, there will be little structural difference between the pre-enamel, Tomes's processes, and cytoplasm of the ameloblasts. An attempt to illustrate some of these features has been made in fig. 3.

Striae of Retzius are common to both mammalian and reptilian enamels, yet their significance is uncertain. It has been shown that the final heavy

calcification of mammalian enamel advances as a front which is actually perpendicular to the striae (Diamond and Weinmann, 1940) and, moreover, in both reptiles and mammals these zones can be recognized in the matrix a considerable time before the onset of final calcification. In view of these facts it is now believed that the striae are related to matrix production rather than to calcification. However, there is evidence that the mineral concerned in the initial influx is derived from the ameloblasts (Marsland, 1951). Unless the ameloblasts can produce matrix proteins and assimilate calcium salts simultaneously, these two processes must be separated in time. This would be achieved if, after producing a layer of matrix, the ameloblasts temporarily suspended the production of organic material and began to assimilate and secrete mineral instead. The cells would thus carry on their two separate functions alternately. The periodic change in the rate of matrix production would account for the presence of the striae of Retzius, which, in reptiles, appear as slight periodic differences in staining properties and refractive index along the matrix fibres. In mammals the striae seem to be the result of regular slight displacements of the prisms (Gustafson, 1945) and this may also be due to the periodic suspension of the normal process of matrix production. On the whole, the zones of normal matrix between the striae of mammalian enamel are much wider than in reptiles, so that the time required to mineralize one zone and convert it into a basophil form is correspondingly greater. This might account for the fact that an acidophil pre-enamel region exists in the matrix of many mammals, but is missing from reptiles where the thin matrix zones can be converted rapidly.

Results obtained with *Agama* are only sufficient to indicate that the final maturation of reptilian enamel follows approximately along the same lines as in mammals. Features noted include the firm re-attachment of the terminal membrane to the matrix, the general shortening of the ameloblasts and the residual matrix becoming acid-soluble. A similarity in the final crystallization processes is suggested by the fact that adult reptilian enamel consists of hydroxyapatite with a crystallite size similar to that of mammalian enamel but approximately ten times greater than that of mammalian or reptilian dentine (Little and Poole, unpublished results). This contrasts with the mesodermal enamel of lower vertebrates which has a crystallite size similar to that of dentine (Poole, 1956a).

ALKALINE PHOSPHATASE DISTRIBUTION

As shown in fig. 2, E, the enamel organ and pulp cavity are sites of considerable phosphatase activity. However, the ameloblasts remain unstained except for the nucleus and the supranuclear or basal cytoplasm (see Symons, 1955), which appear grey. It was found that if prolonged periods of incubation are used, up to 24 h for example, the basal cytoplasm and nucleus of each ameloblast stains quite intensely and this could be due to a gradual diffusion of enzyme from the surrounding enamel organ. Such a diffusion has been shown to occur in mammals during both fixation (Lison, 1948) and incubation

Martin and Jacoby, 1949), but it is also possible that the nucleus is behaving as a slowly acting precipitation centre (Johansen and Linderstrøm-Lang, 1953), the amount of phosphate produced thereby depending upon the time available for the precipitation to occur. Whatever the correct explanation may be, it seems unlikely that the ameloblasts possess intrinsic alkaline phosphatase at this point in their history.

The inner enamel epithelium at the base of a germ always stains intensely. Here, new epithelial cells are being formed by division and these will eventually differentiate into ameloblasts as the formation of enamel and dentine extends downwards from the tip. Exactly the same condition is found in mammalian germs; because of this and the absence of phosphatase from the fully formed ameloblast, it has been suggested that, at this stage, phosphatase may be related more to cell differentiation and growth, or to the manufacture of nucleic acids and other materials, rather than to calcification (Symons, 1955).

Nevertheless, in mammals, phosphatase activity is shown throughout the cytoplasm of the ameloblasts during maturation (Symons, 1955) and, furthermore, phosphatase can be detected in the ameloblasts of fish during the calcification of mesodermal enamel (Kerr, 1955). This evidence has been taken to indicate that alkaline phosphatase is concerned with the process of calcification. However, histological evidence suggests that mammalian ameloblasts are not responsible for the final calcification and that their function is to remove organic material and water from the calcifying matrix (Marsland, 1952). Perhaps alkaline phosphatase is related to the latter function, but further evidence is clearly needed. Unfortunately, because the hardness of the dentine makes section cutting impossible without prior decalcification, it has not yet been possible to discover whether phosphatase is present in the ameloblasts during the maturation of reptilian enamel.

In *Agama* no general staining of the odontoblasts was ever observed; the inner ends of these cells always stained black, so that it is tempting to suggest that diffusion has also occurred here, in this case from the pulp. However, recent results using azo-dye coupling techniques indicate that mammalian odontoblasts possess intrinsic phosphatase of their own (Symons, 1955) and the significance of the results obtained here is therefore difficult to assess. A feature of interest in the reptilian germ is that the parts of the dentinal tubules passing through calcifying dentine nearly always stain black, whereas the parts of the same tubules passing through the uncalcified pre-dentine remain unstained. Clearly, then, during the calcification of the dentine the contents of the dentinal tubules have the ability to precipitate phosphate either by phosphatase enzyme or by some other means.

Finally, mention must be made of the appearance of enamel and dentine after the phosphatase tests have been made. As shown in fig. 2, F, enamel is intensely black whilst the dentine is only light grey. In controls which have been subjected to the last part of the test only—the precipitation of phosphate as cobalt sulphide—exactly the same pattern of black enamel and grey dentine

is produced, indicating that such phosphate is that laid down by the germ naturally. Despite the fact that it stains only lightly, the dentine must possess a considerable amount of mineral, for it is already very hard and brittle, and readily cracks on sectioning. Moreover, it can be made to turn black by prolonged exposure to a cobalt solution. Probably, therefore, the dentine mineral is in the fully formed hydroxyapatite condition and since the exchange of Ca^{++} ions and Co^{++} ions in this mineral is sluggish (Johansen and Lindström-Lang, 1953), complete ionic exchange can only occur after a relatively long period of exposure to a cobalt solution. On the other hand, enamel becomes black after only a few minutes' exposure, suggesting that the mineral here is in a simpler form and is possibly related to the mineral produced under the influence of enzyme in the phosphatase technique. These results confirm the suggestion made earlier that the young basiphil enamel matrix possesses calcium phosphate in some elementary form.

Thus, the behaviour of the mineral involved in the first influx is different from that involved in the final calcification. In the latter process, apparently under the influence of the matrix, mineral crystallizes into orientated hydroxyapatite units of a characteristic size; yet the mineral concerned in the initial influx remains in an elementary condition. Presumably, therefore, during its basiphil stage the matrix undergoes some sort of modification, as a result of which the matrix fibres are rendered capable of exerting an influence over the final calcification of mineral. The striae of Retzius have been shown here to become increasingly apparent as the age of the matrix advances; this might also be taken to indicate the occurrence of some sort of progressive change in the matrix before final calcification begins.

OPTICAL PROPERTIES OF THE MATRIX

The properties described below were found in enamel matrix at all stages irrespective of the fixative used. When a tooth section is examined in water between crossed nicols, both enamel and dentine light up as the surface of the tooth is rotated into the 45° position. If a sensitive tint (first order red) quartz plate is introduced with its slow axis parallel with the tooth surface, enamel appears yellow and dentine blue-green. The birefringence of the enamel is therefore, negative with respect to the tooth surface, or positive with respect to the axis enamel fibres which are perpendicular to the surface. The positive birefringence of the dentine with respect to the surface indicates that, as in many vertebrates (Poole, 1956*a, b*) the positive collagen fibres are orientated parallel with the surface of the tooth.

A significant difference in the behaviour of the enamel and dentine matrices was found when sections were mounted in aqueous phenol solution and re-examined as above. Under these conditions both the enamel and dentine are yellow, indicating that the sign of birefringence of the dentine has been reversed. This reversal in phenol solution is a property of collagen not shared by other skeletal proteins (Frey-Wyssling, 1953), and is to be expected in dentine but not in enamel if the latter is keratinous. However, the optical

properties of the enamel matrix were found to depend very much upon the refractive index of the solution in which sections were mounted. A summary of the properties in various mounting liquids is given in table 1.

TABLE 1

<i>Liquid</i>	<i>Refractive index</i>	<i>Birefringence of enamel matrix relative to fibre axis</i>	<i>Becke line test</i>
Water . . .	1.33	positive and strong	R.I. enamel > 1.33
Ethyl alcohol . . .	1.36	positive	R.I. enamel > 1.36
Butyl alcohol . . .	1.40	positive	R.I. enamel > 1.40
Benzene . . .	1.50	positive but faint	R.I. enamel > 1.50
Clove oil . . .	1.53	positive but very faint	R.I. enamel > 1.53
Mixture of clove oil and bromonaphthalene .	1.57	isotropic	R.I. enamel = 1.57 enamel invisible
Bromonaphthalene .	1.66	positive	R.I. enamel < 1.66
Methylene iodide . .	1.76 ⁸¹	positive and strong	R.I. enamel < 1.76

By means of the Becke line test the refractive index of the enamel matrix was found to be about 1.57; young matrix was very slightly less than this, and mature matrix slightly greater. It may be seen from the table that the fibrous matrix has no birefringent properties when it is mounted in a liquid with a similar refractive index of 1.57. Birefringence is only produced by mounting in liquids whose refractive indices are either significantly greater or smaller than 1.57, with maximum birefringence produced at the extremes (1.33 and 1.76). All this points to the fact that the matrix fibres have no intrinsic birefringence; but since they are parallel with each other they form, together with the mounting liquid, a Wiener mixed body. Such a system produces a form-birefringence positive with respect to the direction of orientation of the micelles, the magnitude depending upon the difference between the refractive indices of the two components. If these two indices are the same, the system is optically isotropic.

The behaviour of the enamel matrix in paraffin wax is very interesting. During the examination of newly prepared sections before the wax had been removed, it was found, even under these conditions, that the matrix showed not only distinct activity but also, rather surprisingly, a birefringence which was negative with respect to the fibre axis; that is, opposite in sign to the form-birefringence described above. As the wax is dissolved away in a solvent, the negative birefringence is replaced by the positive form-birefringence. These properties were investigated further by taking wax sections and heating the slide until the wax became molten. In the molten medium the fibrous matrix showed, as in any other liquid, the typical positive form-birefringence; but as soon as the wax solidified the negative birefringence reappeared. It was possible to repeat these reversals indefinitely by alternately warming and cooling the slide.

The explanation of this phenomenon is probably to be found in the

properties of the crystallized paraffin. If paraffin molecules are orientated parallel with each other (e.g. by flow or crystallization), the system shows a birefringence which is positive with respect to the direction of orientation. Paraffin wax crystals are flat plates with the paraffin molecules perpendicular to the plane of the plate. These plates may be joined together, edge to edge, to form elongated flat lamellae which appear needle-like when seen in side view. In such lamellae the wax molecules are again perpendicular to the plane

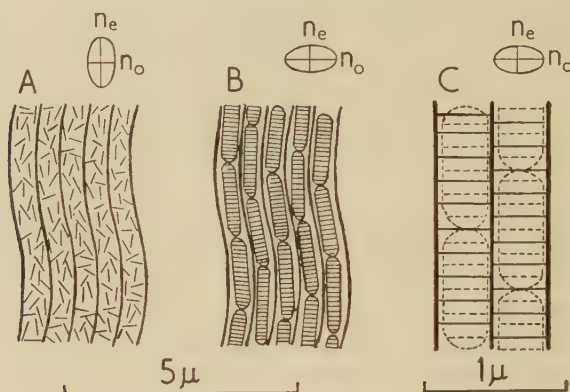


FIG. 4. Diagram of the suggested submicroscopic appearance of reptilian enamel matrix in paraffin wax. The index ellipse of each system is represented by the axes n_e and n_o . A, in molten wax, randomly scattered paraffin molecules lie between the parallel fibres producing a positive form birefringence with respect to the fibre axes. B, the arrangement of elongated paraffin streaks resulting from the crystallization of the wax. Because of the orientation of the paraffin molecules, a negative intrinsic birefringence is set up. C, a more detailed plan of the association between matrix and crystallized paraffin. Parallel enamel fibrils are joined by perpendicular cross members; the latter are responsible for the primary alignment of paraffin molecules, as a result of which the negative streaks lie parallel with the vertical fibrils. Fibrils and cross members are represented by continuous lines, paraffin molecules and streaks by broken lines.

of the lamella and also perpendicular to the axis of elongation of the lamella. Thus, in side view, the lamella exhibits a negative birefringence with respect to the longitudinal axis and because of this is known as a 'negative streak' (Frey-Wyssling, 1953). These facts can now be applied to the behaviour of wax in enamel matrix. In molten form the paraffin wax molecules will be randomly scattered and so the medium behaves normally as a liquid to produce a positive form-birefringence. As the wax crystallizes, negative streaks are formed which become orientated parallel with the enamel fibres. Thus the positive form-birefringence becomes replaced by the negative intrinsic birefringence of the paraffin streaks as crystallization occurs. These properties are illustrated in fig. 4, A, B.

The method by which the negative streaks become orientated is a matter of speculation. It may simply be a mechanical process with the paraffin streaks tending to slip into the spaces between the fibres, but other properties of paraffin crystallites suggest that the mechanism of orientation may be more

complex than this. In strips of cold-drawn polyethylene it is found that the molecules become orientated approximately parallel with the direction of drawing. If paraffin wax is allowed to crystallize on the surface of such strips the paraffin molecules become aligned parallel with the polyethylene molecules with the result that the crystal plates of paraffin are arranged with the plane of the plate perpendicular to the direction in which the polyethylene strips are drawn (Richards, 1951). Similarly, in spherulites of polyethylene where molecules are arranged tangentially, molecules of crystallizing paraffin again become parallel with those of the polyethylene with the result that the paraffin streaks are arranged radially across the concentric layers (Wilems and Wilems, 1956). From this it is evident that the arrangement of paraffin streaks is secondary since it depends upon the initial direction in which the constituent molecules become orientated.

A similar mechanism may determine the arrangement of paraffin streaks in enamel matrix. If so, the matrix must possess some sort of cross structures running at right angles to the fibres. Such structures would determine the primary orientation of the paraffin molecules perpendicular to the fibres axes and would result in the secondary arrangement of negative streaks parallel with the fibres, as suggested in fig. 4, c. The coarse fibrous appearance of the enamel matrix is, perhaps, only the visible manifestation of a more delicate submicroscopic pattern. The latter would need to possess two main components; first, fibrils lying parallel with each other and at right angles to the tooth surface which set up the positive form-birefringence; secondly, side groups lying perpendicular to and possibly connecting the fibrils together. The whole system thus forms a lattice grid, the cross members of which determine the orientation of paraffin molecules. It has been shown that the crystal structure of polyethylene is very closely similar to that of paraffin wax (Bunn, 1939), and is therefore ideal for the orientated overgrowth of crystals (Wilems and Wilems, 1956). If the above suggestions are correct, the side groups running between the fibrils in the organic matrix may also possess a crystal structure related in some way to that of paraffin wax.

The orientation of crystals by organic fibres is of special interest in the study of calcified tissues, for in bone, dentine, and mesodermal enamel (Schmidt, 1938, 1940; Poole, 1956*a*), and in reptilian enamel as described here, crystallites of hydroxyapatite are arranged with their crystal *c* axes parallel with the organic fibres axes. Recent electron microscope studies (Jackson and Randal, 1956; Fernández-Morán and Engstrom, 1956) have made it possible to 'see' the intimate relationship between mineral crystallites and organic fibres, but the precise mechanism of mineral orientation, whether or not it involves crystal overgrowth, is still obscure.

DISCUSSION

The evidence presented here indicates that the process of enamel formation in reptiles is essentially the same as the corresponding process in mammals. The main difference is that whereas developing reptilian matrix is continuously

fibrous, mammalian matrix is broken up into units, the prisms, each one having been produced by one ameloblast and isolated from its neighbours by interprismatic material. Nevertheless, the fibres of young reptilian matrix have been shown to be arranged in groups which correspond with the ameloblasts; the production of new material to separate the groups from each other would result in a condition very similar to that found in mammals and, presumably, this is the most important change which took place in the evolution of the mammalian type of enamel from reptilian.

It has been suggested that the terminal bar apparatus in mammals may be associated with the production of interprismatic material (Marsland, 1951), so that it is of special interest to note that these heavy intercellular condensations at the bases of the ameloblasts have not been found in the reptiles where the enamel lacks prisms. The transition of prismatic enamel from the homogeneous reptilian type, therefore, may have been achieved by the development of the terminal bar apparatus, which, by the production of a material differing somewhat from the matrix proper, separates the products of the ameloblasts into prisms.

As regards structural properties, it must also be noted here that although mammalian matrix shows no fibres even when examined with the electron microscope (Little, 1956), it has, nevertheless, a faint but definite positive birefringence with respect to the axes of the prisms (Schmidt, 1934; Keil, 1937). This property has been taken as an indication of an organized fine structure within the matrix which might be responsible for the orientation of mineral crystallites parallel with the prisms. However, it is clear that the orientated, possibly molecular, units producing the birefringence must be of very narrow width if they are beyond the resolution of the electron microscope. Consequently, the second important change in the evolution of prismatic enamel was the production of a more refined and delicate matrix instead of the coarse fibrous type characteristic of reptiles. As has already been pointed out, the fibrous appearance of reptilian matrix may only be the outward manifestation of a more intricate submicroscopic structure.

The evolution of prismatic enamel from reptilian seems, therefore, to have been a comparatively simple step, and tubular enamel, found in most marsupials and a few placentals, must be considered to be a later specialization of the simpler prismatic type. Tubular enamel possesses both prisms and tubules, the latter arising at the amelodontinal junction in the so-called 'clumsy' joint. The origin of these tubules is still not clear, but they are known to be interprismatic and may be occluded by the deposition of calcium salts in older enamel (Sprawson, 1930). Although X-ray analysis shows the presence of hydroxyapatite crystallites orientated approximately parallel with the prism axes, as in normal prismatic enamel, marsupial enamel exhibits most unusual optical properties when treated with dehydrating and clearing agents (Poole, 1952). These properties may be in some part due to the high organic content of the enamel. The possession of prisms shows that tubular enamel must be related to the more normal mammalian type, but the tubules, the

peculiar optical properties, and the relatively high organic content all point to a specialized type of prismatic enamel.

In higher vertebrates, therefore, three distinct types of enamel matrix exist, all of which result from the calcification of a matrix derived from the ectodermal ameloblasts. Similarly, in lower vertebrates, different types of enamel are to be found with the common feature of being formed in a matrix derived from the mesodermal odontoblasts (Poole, 1956a). The phylogenetic relationships between mesodermal and ectodermal enamels have been discussed by Kvam (1946, 1953), who considers the first step in the transition from one to the other to have been the production of a tissue jointly by mesoderm and ectoderm. In general, it has been tacitly assumed that both the production and the calcification of mammalian matrix are performed by the ameloblasts, and the suggestion has repeatedly been made that even in fish the ameloblasts may furnish the mineral required for the calcification of the mesodermal matrix (Tomes, 1898; Kvam, 1946, 1953; Kerr, 1955). If this is so, the only further requirement for the evolution of an ectodermal enamel is the ability of the ameloblasts to produce an organic matrix.

However, evidence that has gradually accumulated over a number of years suggests strongly that much of the mineral involved in the calcification of mammalian enamel is derived from the dental pulp (see Marsland, 1952). Marsland himself came to the conclusion that, although the initial mineral influx originates from the ameloblasts, none of the calcium salts required for the final calcification comes from these cells and their function during maturation is to withdraw organic material and water from the calcifying matrix. Thus, there is a double source of mineral salts for the calcification of mammalian enamel and the phylogenetic transition suggested above is not quite so easy to accept. In fact, no hypothesis can be regarded as satisfactory until the source of the mineral used in the calcification of the mesodermal enamel has been clearly established.

Finally, brief reference may be made to the problem of the organizing activities of tooth-tissues. In mammals it is generally believed that as soon as the enamel organ and dentine papilla are completed, the ameloblasts produce an organizer which induces the odontoblasts to begin dentine formation; later, the ameloblasts themselves are induced by the odontoblasts to produce enamel. In lower vertebrates also it is possible that the ameloblasts organize the activities of the odontoblasts, but the induction of the ameloblasts by the odontoblasts is lacking and, presumably, this is a necessary condition for the production of a wholly ectodermal enamel. There appears to be no direct evidence for such an organizer from the dentine papilla even in mammals; nevertheless, in the somewhat similar process of feather production, where mesodermal and ectodermal tissues also work in close association, there is evidence that the mesodermal papilla induces activity in the ectodermal cap, culminating in the formation of a feather (Wang, 1943). This is regarded as an example of a 'secondary' organizer acting at a late stage in the life history of an animal (Waddington, 1956), and it is tempting to suggest that the

induction of ameloblastic activity by the dentine papilla, which also occurs at various stages in life, may be a parallel process.

It is hoped that, as a result of this account of the formation and properties of reptilian tooth-tissues, some of the relationships between the various types of vertebrate enamel are a little clearer. Much of the discussion has necessarily been speculative, but further research may solve many of the outstanding problems, such as the points of origin of the first type of ectodermal enamel and of the later prismatic type. The study of the teeth of primitive mammals and their possible reptilian ancestors would be of great value, and it is hoped that such material may sometime become available.

In conclusion, the author would like to express his gratitude to the Research Grants Committee, Makerere College, for providing funds for the purchase of a polarizing microscope; to Professor A. I. Darling, University of Bristol Dental School, for a number of helpful comments on the work undertaken; and to Professor L. C. Beadle, Zoology Department, Makerere College, for reading and criticizing the manuscript.

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The Uptake of ^{35}S in Cortical Bone

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With three plates (figs. 1-3)

SUMMARY

A further study of the uptake of ^{35}S in rabbits and in normal and rachitic puppies has been undertaken. The non-labile ^{35}S present in osteoid tissue is removed by testicular hyaluronidase, but not by staphylococcal hyaluronidase, which suggests that it is chondroitin sulphate A or C. The non-labile ^{35}S is first taken up by non-calcified osteoid tissue in the area giving a pale pink PAS reaction. It is only later found in calcified bone when the tissue ceases to be PAS-positive. Rigid attention to technical detail is essential if comparable results are to be obtained in studies of bone uptake of ^{35}S .

INTRODUCTION

EXPERIMENTS already reported (Kent, Jowsey, Steddon, Oliver, and Vaughan, 1956) on the uptake of ^{35}S given by intravenous injection to rabbits suggested that ^{35}S was taken up in two forms in cortical bone: (i) a labile form throughout the bone which is in large part removed by decalcifying agents; (ii) a non-labile form not removed by decalcifying agents. This was shown by chemical analysis to be chondroitin sulphate or a closely allied substance (Kent and others, 1956) and is probably associated with the ground substance of the bone matrix (Bélanger, 1954; Vincent, 1954; Leblond, Bélanger, and Greulich, 1955).

The present study was undertaken in order to determine

- (i) the effect of fixatives and embedding materials on the character of the ^{35}S incorporated in bone matrix as evidenced by reaction to hyaluronidase;
- (ii) the effect of hyaluronidase from different sources on ^{35}S incorporated in bone matrix;
- (iii) the exact location of non-labile sulphate in bone matrix at different times after injection.

EXPERIMENTAL PROCEDURE

Animals. The animals investigated were rabbits 6-8 weeks old, normal puppies 6-9 weeks old, and normal and rachitic puppies 26 weeks old. Rickets was produced as described in previous experiments (Jowsey, Sissons, and Vaughan, 1956) by the use of a rachitogenic diet devised by Mellanby (Mellanby, 1950). The animals were killed at various times after the injection of ^{35}S as $\text{Na}_2^{35}\text{SO}_4$ (2,000-3,000 $\mu\text{C/kg}$), and pieces of cortical bone, 3 cm long, were taken from the tibiae.

Microradiographs were prepared by methods already described (Owen,

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Jowsey, and Vaughan, 1953; Jowsey, 1955) from sections from bones of all the animals in the experiment in order to check the degree of calcification present.

Autoradiographs were made from sections prepared by different methods described below. The section was attached to a slide with a solution of collodion in ether-alcohol. The film was floated on to the section from a bath of distilled water containing 10% glycerine at 28° C. The films were thoroughly dried in a current of air from an electric fan and the sections stored in light-tight boxes in a refrigerator. The time of exposure varied according to the dose/kg the animal had received, and the time which had elapsed between injection and exposure. The autoradiographs were developed for 5 min in D19b (Kodak) at 18° C, fixed in 'hypo' for 2.5 min, and washed well. In some cases after fixation the autoradiograph was floated off the section and remounted. Fast experimental film (Kodak Scientific Plate No. V 1001) was used in the preparation of autoradiographs from bone sections containing ³⁵S (Kent and others, 1956). The resolution given with this film is poor and there is much background fogging, but the uptake of ³⁵S in the skeleton is low. The maximum retention previously found in the femur 7 days after injection was 0.02% of the injected dose; therefore there is little ³⁵S present in any one section. The half-life of ³⁵S is only 87 days and if the sections have to be exposed twice it is essential to obtain a quick result. For purposes of comparison, photomicrographs have been included of autoradiographs of cross-sections of the tibiae from rabbits and puppies in the same age groups used in other experiments when ⁹⁰Sr was given (Jowsey, Sissons, and Vaughan, 1956). ⁹⁰Sr is taken up in mineralized bone and not in osteoid (Vaughan, 1956).

HISTOLOGICAL METHODS

Preliminary studies suggested that the effect of hyaluronidase on ³⁵S incorporated in bone matrix is influenced by methods of fixation and embedding. Conditions must be constant if consistent results are to be obtained. Cross-sections were therefore prepared from pieces of cortical bone in the following ways:

(i) The tissue was fixed in absolute alcohol, embedded in methyl methacrylate monomer, and cross-sections were cut and ground to approximately 50 μ (Jowsey, 1955). The sections were immersed in chloroform for about 18 h to remove the monomer; they were hydrated, washed in distilled water, and then demineralized in EDTA for 24 h. The decalcified sections were then mounted.

(ii) The tissue was fixed in absolute alcohol for 3 days, during which the alcohol was changed 3 times. Pieces of bone were then decalcified in EDTA as previously described (Kent and others, 1956), and washed in running water for 1–2 h.

(a) The piece of bone was placed in ether-alcohol for 2 days; the ether-alcohol was changed once. The piece was then put in 1% collodion

solution (BDH) for 1 day, then in 4% collodion solution for 1 day, and then embedded in 6% collodion and allowed to set in a partial vacuum. When set, sections 5–10 μ thick were cut from the blocks on an MSE microtome. Collodion was removed from the sections by immersion in ether-alcohol for 48 h. The sections were taken down through the alcohols to 70% alcohol and mounted.

- (b) Other pieces of bone were dehydrated (70% alcohol for 2 h, 90% for 2 h, absolute for 3 changes of 4 h). They were then cleared in benzene (two changes of 4 h each), followed by three changes of increasing strengths of wax (melting-point 58°C) mixed with benzene at 60°C each for 2 h and then the block was cast with wax of the same melting-point. Sections were cut (5–10 μ) and mounted; the paraffin wax was removed from the sections with xylene and the sections were then taken down through the alcohols to 70% alcohol.
- (iii) The tissue was fixed in neutralized formaldehyde-saline for 3 days and then embedded in
 - (a) collodion as described;
 - (b) paraffin as described.
- (iv) The tissue was fixed in formaldehyde-saline at pH 9 and embedded in
 - (a) collodion;
 - (b) paraffin.

Some of these sections were washed extremely well and others less well before preparation of autoradiographs.

TREATMENT WITH HYALURONIDASE

Some sections prepared in all the different ways described above after the first autoradiograph was floated off were treated for 6 h at 37°C with 0.1% testicular hyaluronidase (Evans Rondase) buffered at pH 5.6. The sections were then remounted and further autoradiographs made, a correction being allowed for the decay of ^{35}S in calculating exposure time. Other sections fixed in absolute alcohol and embedded in monomer and sections fixed in neutral formaldehyde-saline and embedded in paraffin were treated with staphylococcal hyaluronidase after the first autoradiographs were floated off. This hyaluronidase was buffered at pH 6 and incubated at 37°C for 6 or 23 h. Each ampoule contained 160 turbidity-reducing units of enzyme, i.e. about 4 times as much as an ampoule of Evans Rondase. This was dissolved in 1.0 ml of 0.2% gelatin containing $2 \times 10^{-4}\text{M}$ sodium pyrophosphate adjusted to pH 7 to guard against metal inactivation. Some sections treated with appropriate buffer solutions and without hyaluronidase were also used for the preparation of autoradiographs.

RESULTS

The effect of different fixatives and embedding material

The effect of variation in fixation and embedding on the action of testicular hyaluronidase in removing ^{35}S from bone matrix are shown in table 1.

Testicular hyaluronidase removed ^{35}S from sections embedded in monomer or collodion but not from sections embedded in paraffin. The same results were obtained in material fixed in absolute alcohol, neutral formaldehyde-saline, or formaldehyde-saline at pH 9.

The effect of testicular and staphylococcal hyaluronidase

Testicular hyaluronidase removed all ^{35}S from both normal and rachitic osteoid except when the sections were embedded in paraffin. Staphylococcal hyaluronidase failed to remove ^{35}S from both paraffin and monomer embedded sections of normal bone. It was not used on rachitic bone. Buffer solutions at pH 5.6 and pH 7 alone were without effect on ^{35}S uptake.

TABLE I

Effect of fixation and embedding technique on action of hyaluronidase

Technique	Testicular hyaluronidase
Absolute alcohol, monomer	+
Absolute alcohol:	
(a) collodion	+
(b) paraffin	O
Neutral formaldehyde-saline:	
(a) collodion	+
(b) paraffin	O
Formaldehyde-saline pH 9, well washed:	
(a) collodion	+
Formaldehyde-saline pH 9, poorly washed:	
(a) collodion	+

+ = ^{35}S removed; no reaction in 2nd autoradiograph.

O = ^{35}S not removed; reaction present in 2nd autoradiograph.

Location of non-labile ^{35}S

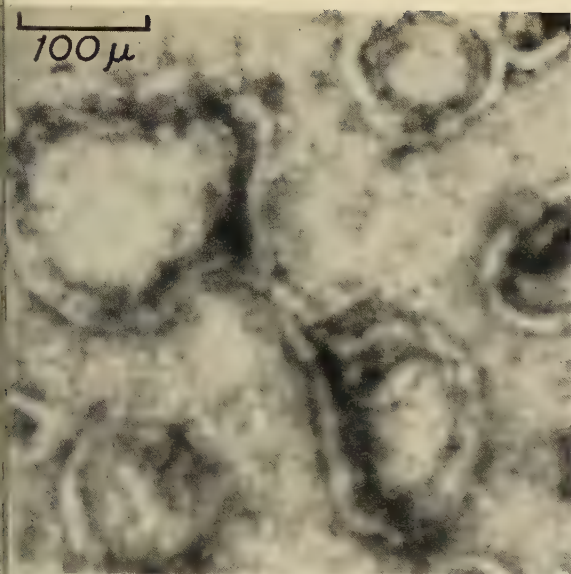
In animals killed 3 h after injection. Autoradiographs of cross-section of cortical bone from the normal puppy showed ^{35}S on the edge of the osteoid seam of building sites, in the area giving a positive PAS reaction in alcohol-fixed non-decalcified material (fig. 1, A, B). This pattern of uptake of ^{35}S over the osteoid seam is markedly different from that seen for ^{90}Sr in a dog killed at the same time after injection when the ^{90}Sr appears in calcified bone behind the osteoid seam (fig. 1, C). ^{35}S was also found in the outer lamellae of periosteal and endosteal bone, but it was less easy here to be certain that it was present only on the extreme periphery of the seam since calcification in these sites is always more rapid than in internal osteones.

FIG. 1 (plate). Autoradiograph left on decalcified section from tibia of 6-month-old dog killed 3 h after injection with ^{35}S (2,000 $\mu\text{C}/\text{kg}$).

A, osteoid border in focus.

B, grains of autoradiograph in focus. Note heavy uptake of ^{35}S over extreme edge of osteoid border; heavy background fogging.

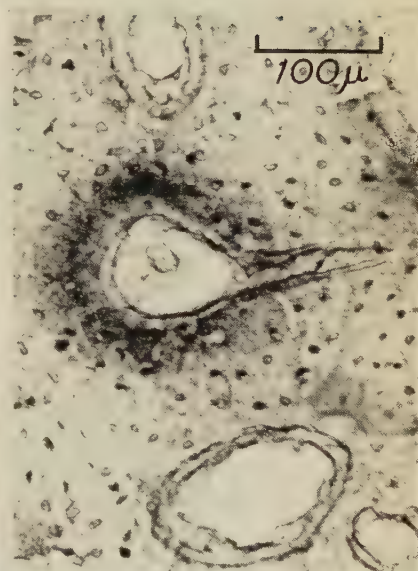
C, autoradiograph left on non-decalcified section from tibia of 5-month-old dog killed 3 h after injection of ^{90}Sr (500 $\mu\text{C}/\text{kg}$). Note heavy uptake of ^{90}Sr behind osteoid border.



A



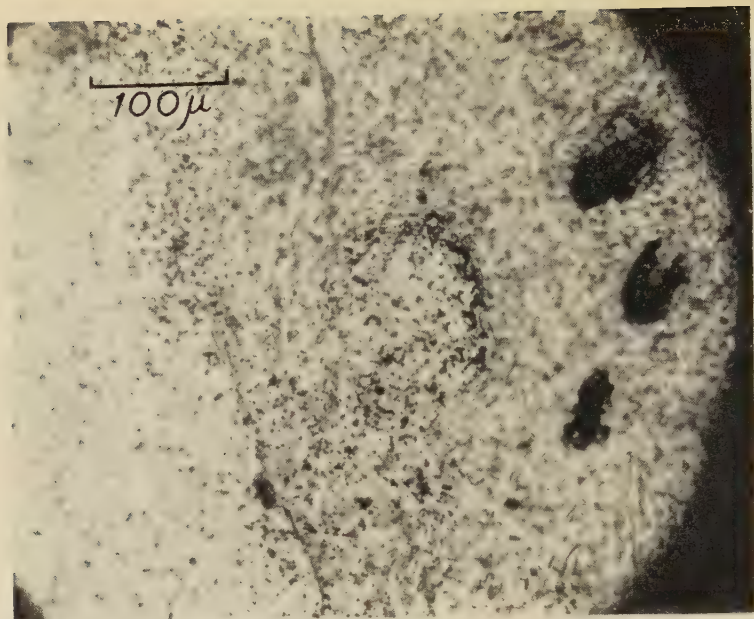
B



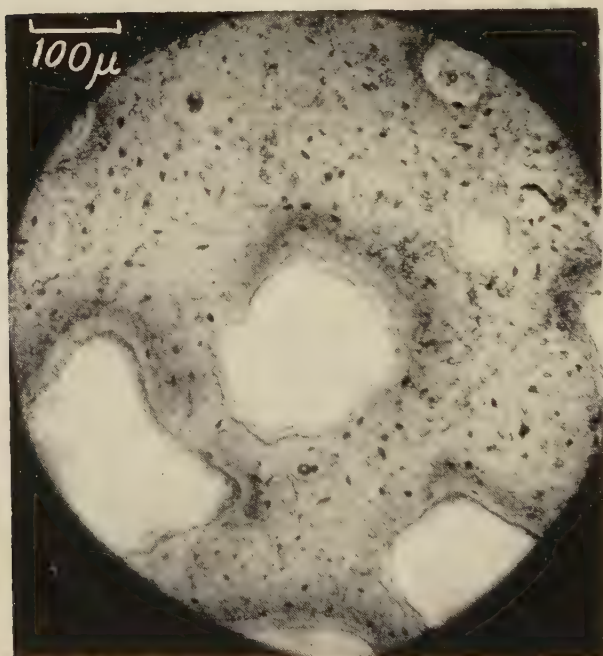
C

FIG. 1

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A



B

FIG. 2

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In animals killed 24 h after injection. There was rather more ^{35}S seen after decalcification than in the animal killed after 3 h. It was then present over the whole of the osteoid seam in active building sites (fig. 2, A) and in a thin line in the recently formed periosteal and endosteal bone. In the case of ^{90}Sr the radioactive isotope is again seen behind the osteoid border (fig. 2, B).

In animals killed 5 and 7 days after injection. The reaction in autoradiographs spreads back behind the osteoid seam into calcified bone (fig. 3, A, B). In periosteal and endosteal areas the reaction could be seen in a well-marked band with bone both internal and external to it showing no reaction. In the case of ^{90}Sr (fig. 3, C) there is a clear band of non-radioactive calcified bone between the osteoid seam and the bone containing ^{90}Sr .

The osteoid of rickets

Preliminary radiochemical estimations suggest there may well be quantitative differences in the uptake of ^{35}S in normal and rachitic osteoid. These studies are to be extended. Autoradiographs showed that ^{35}S was taken up in rachitic bone in the same sites as in normal bone, i.e. in areas where new osteoid tissue was being laid down.

DISCUSSION

It is not at present clear why, if tissue is embedded in paraffin, hyaluronidase fails to remove ^{35}S . It is, however, important to recognize that this is so, and to follow a constant technique in histological study of ^{35}S uptake.

It has already been shown by chemical analysis (Kent and others, 1956) that the radioactive material extracted from decalcified bone after injection of ^{35}S is chondroitin ^{35}S sulphate or some closely allied substance. The fact that negative autoradiographs are obtained from decalcified sections of bone fixed in alcohol and embedded in monomer after treatment with testicular hyaluronidase, but not after staphylococcal hyaluronidase, supports the view that at least the greater part of the ^{35}S fraction is chondroitin sulphate A or C (Kent and Whitehouse, 1955; Meyer and Rapport, 1951; Meyer and others, 1956). Both chondroitin sulphate A and C are hydrolysed by testicular but not by bacterial hyaluronidase, while B is hydrolysed by both. Both A and C have been isolated from bone, though B has not (Meyer and others, 1956). Further work on the other characteristics such as optical rotation and solubilities of the material taking up ^{35}S will be required to discriminate between chondroitin A and C. It is possible, however, that there are in bone extremely small quantities of other substances which may be sulphated (Meyer and others, 1956). The ^{35}S present in the first lamella of the osteoid border of a building site probably is in a different chemical form to the ^{35}S present later in calcified tissue. In the former site the tissue is coloured a good pink with PAS in

FIG. 2 (plate). A, autoradiograph of decalcified section from 6-month-old dog killed 24 h after injection of $3,000\text{ }\mu\text{C/kg}$ ^{35}S . Note uptake over osteoid border but not at extreme edge as in fig. 1.

B, autoradiograph left on non-decalcified section of tibia from dog killed 24 h after injection with ^{90}Sr ($600\text{ }\mu\text{C/kg}$). Note uptake of ^{90}Sr well behind osteoid border.

alcohol-fixed, undecalcified material, while 21 days later, or even 7 days later, the tissue containing the ^{35}S shows no distinctive PAS reaction, suggesting that ^{35}S is present in a different chemical combination. Leblond, Bélanger, and Greulich (1955) working with rats have shown a similar process occurring in periosteal bone, i.e. the deposition first of ^{35}S in non-mineralized osteoid followed by mineralization at a slightly older level of matrix. It has, indeed, been suggested by many workers that the chondroitin sulphate of the ground substances acts in some way as the means by which the mineral apatite crystals are associated with the collagen fibres (Kent and Whitehouse, 1955; Kent and others, 1956).

It should be noted that there are other areas in bone which give a positive PAS reaction but which do not take up ^{35}S . This may be due to metabolic inactivity of the tissue, which is probably the case in cartilage remnants or to the fact that the reacting material does not contain sulphur but one of the other mucoproteins known to occur in bone (Hismura, 1938; Eastoe and Eastoe, 1954; Glegg, Eidinger, and Leblond, 1954; Glegg and Eidinger, 1955).

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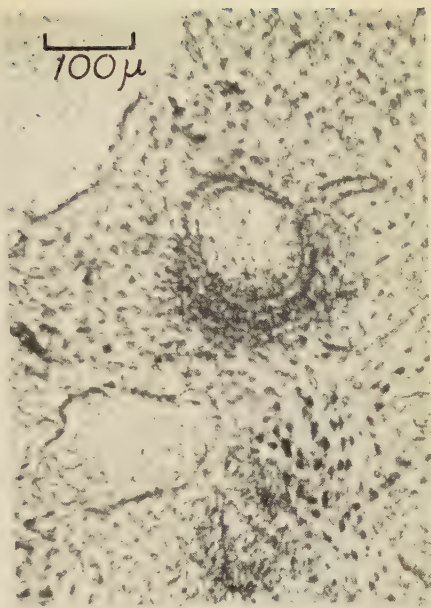
FIG. 3 (plate). A, autoradiograph left on decalcified section from tibia of 9-week-old dog killed 7 days after injection with ^{38}S . Note uptake behind osteoid border.

B, microradiograph of same section showing that ^{35}S is now in calcified osteoid.

C, autoradiograph left on undecalcified cross-section of cortical bone from tibia of young dog killed 7 days after injection with ^{90}Sr (600 $\mu\text{c/kg}$).



A



B



C

FIG. 3

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